(54) **Method and device for determining location and the number of a fluorescent molecule.**

(57)   One characteristic of this invention is that to
confirm presence of fluorescent molecules, lo-
cations and the number of the fluorescent
molecules in a local area irradiated with exci-
tation light are determined based on the num-
ber of fluorescence photons generated per unit
period of time by the irradiation of the exci-
tation light counted as a quantized fluoresc-
ence intensity of the fluorescent molecules, and
a distribution of appearance frequencies of the
fluorescence photons formed on a two-dimen-
sional image. A typical constitution for conduc-
tion the method comprises a light source for
irradiating excitation light to a local area on a
flat substrate with the adsorbed fluorescent
molecules, and fluorescence detecting means
for detecting fluorescence photons from the
fluorescent molecules, and forming a distribu-
tion of appearance frequencies of the fluoresc-
ence photons per unit period of time on a
two-dimensional image corresponding to the
local area.

Fig. 2

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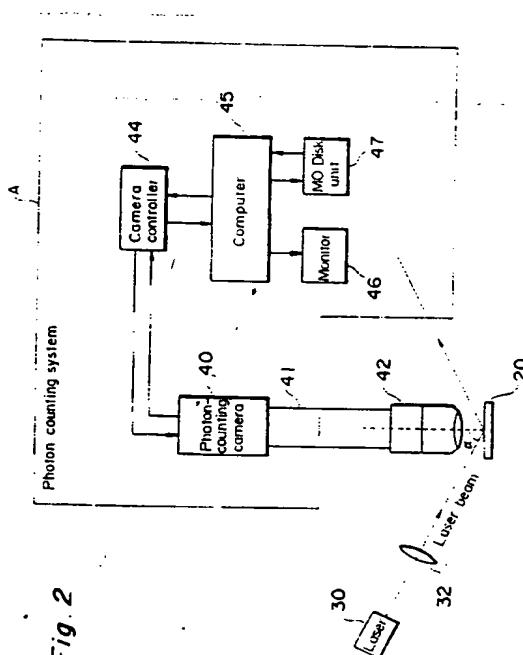
BACKGROUND OF THE INVENTION

Field of the Invention

5   This invention relates to a method and a device for optically discriminating nucleic acid bases (e.g., nucleotide) constituting a gene to determining a sequence of the nucleic acid bases specifically to an art in which basically fluorescence or luminescence of fluorescent molecules represented by the base to discriminate number, position, etc. of the fluorescent molecules.

10   Related Background Art

A DNA (deoxyribonucleic acid containing base as a main component, and sugar and phosphoric acid is bonded to the base), which is a composition material of a gene, has a double helical form. The double helix contains genetic information. Each DNA contains one genetic information in a code-like form (base sequence).
15   Genes are gathered in strings in the cell nucleus. Lower organisms, such as microorganism, have thousands of nucleotide pairs at most, but higher organisms having more genetic information have several billions to 29 billions of base pairs.

Genetic information indicated by DNA, which is a main body of a gene, is determined by a sequence of four kinds of base, i.e., adenine, guanine, cytosine and thymine. Accordingly, it is very significant to know their
20   sequences for the future development of genetic engineering, medicine, etc.

It is known that these base emit their intrinsic fluorescence. Their fluorescence increase especially at low temperatures (100 K or less). It is passible to discriminate the base in principle based on their fluorescence lifetimes. To generate fluorescence, it is necessary to irradiate excitation light. A high sensitivity detector, such as a photomultiplier, is suitably used for the detection of the fluorescence. As an apparatus for detecting a
25   single fluorescent molecule, the apparatus disclosed in, e.g., "Proc. Natl. Acad. Sci.", U.S.A., 86 (1989) 4087-91 (a first conventional method) is known. In this reference, as shown in Fig. 1, excitation light (laser beam) from a light source is applied to a flow cell 61 containing dye solution, and fluorescence is detected by a photomultiplier 63 in a direction which is normal to both directions of irradiation of the excitation light and of flow of the dye solution.

30   In Fig. 1, an optical system for forming an image on the photomultiplier 63 comprises a lens 64, an aperture 65, a wavelength selecting filter 66 and a condensation lens 67. A measuring system for measuring the fluorescence detected by the photomultiplier 63 comprises an electric signal detecting/multiplying unit 68, a fluorescent photon counter 69 and a computer 70.

There is a second conventional method which respective single-fragment bases are modified by a fixable
35   fluorescent dyes (Japanese Patent Laid-Open Publication No. 100945/1991, United States patent No. 4,962,037). The second conventional method has the same arrangement in which the respective bases are labelled by their characteristic dyes, then cut off by exonuclease III, and a sequence of the bases are determined based on differences in fluorescence spectrum.

A third conventional method as another single fluorescent molecule detection relies on high resolution
40   spectroscopy of single impurity aromatic molecule (pentacene) embeded in an organic molecule (paraterphenyl) (J. Chem. Phys. 95(10), 15 Nov. 1991, 7150-7163). This third conventional method is not suitable for detecting the base, but measures a fluorescence excitation spectrum of pentacene at ultra-low temperature (about 4 K) to measure an uniformly wide spectrum in an ununiformly wide spectrum and uses the former as a fluorescence spectrum of the single molecule.

45   On the other hand, advantages of the first conventional method produced by using a flow cell 61 are that degradation of dyes can be suppressed, and that a filter can be provided in a dye circulation system to remove dust. But fluorescence can be observed only in a period of time (about µsec) in which the molecule is passing through a region irradiated with the excitation light irradiated. Accordingly, in the device of Fig. 1, the fluorescence from the base of genes cannot be correctly and efficiently detected; only four kinds of base, A, G, C,
50   and T, are contained in one DNA, and their numbers are very large, but on the other hand, their sizes are very small, and fluorescence generated from their base is very feeble. A detection of fluorescence during an instance in which the beam of the excitation light passes through the base is very difficult to discriminate kinds of the base.

A longer period of time being able to generate the fluorescence and higher efficiency can be obtained by
55   irradiating the excitation light to a base flow over a larger area in the direction of the base flow. But in this case, the base which is fed one after another is simultaneously detected, with the result that processing of the obtained data is difficult.

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SUMMARY OF THE INVENTION

The present invention finally aims at a method for determining a sequence of the bases (A, G, C, and T) constituting a single fragment of nucleic acid. An object of the present invention is to provide a method for speedily and accurately discriminating a kind base including a gene. To realize this method, the present invention provides an optical discriminating device for detecting fluorescence or luminescence to detect fluorescent molecules.

The device for determining a location of a molecule-group and the number of a fluorescent molecule in the molecule-group according to this invention comprises a light source (e.g., a laser device) for irradiating excitation light to a local area on a flat substrate with a group of adsorbed fluorescent molecules fluorescence detecting means for detecting a fluorescence photon generated by irradiating the excitation light to a local area on the substrate, and measuring the number of the fluorescence photon per unit period of time as a quantized fluorescence intensity of the molecule-group.

Here quantization includes "to express as multiples of a definite quantity".

This constitution realizes the method for determining a location of a molecule-group, and the number of a fluorescent molecule in the molecule-group. Characteristics of this constitution are comprise:

Firstly, the fluorescence detecting means is disposed at a position which is out of optical paths of the excitation light from the light source, and of reflected light on the surface of the substrate; and

Secondly, the fluorescence detecting means comprises an optical microscope (variable magnification) for condensing a fluorescence in a local area on the surface of the substrate, and a photon counting system for counting a fluorescence photon condensed by the optical microscope, and displaying an appearance frequency distribution of the fluorescence photon on a two-dimensional image corresponding to the local area.

In particular, the photon counting system forms the appearance frequency distribution of the fluorescence photon in the unit period of time by plotting the frequency of the appearance of the fluorescence intensity in the fluorescent spots on a two-dimensional image of the sample substrate. The number of the fluorescent molecules in the fluorescent spots is identified based on the appearance frequency distribution of the fluorescence photon per the unit period of time displayed on the two-dimensional image.

Furthermore, the device may comprise moving means for relatively moving the local area on the substrate. This moving means comprises an X-Y stage for moving the local area on the substrate. This moving means comprises an X-Y stage which horizontally moves with respect to the fluorescence detecting means, and a rotary stage supported by the X-Y stage for fixing the substrate.

Specimen (substrate) with a molecule-group comprising a fluorescent molecule adsorbed on is prepared by spraying a solution containing the fluorescent molecule in a fine liquid droplet containing the molecule-group, sticking the fine liquid droplets to the surface of the flat substrate, and then drying the substrate at room temperature or less.

At this time, the fluorescent molecule contained in the solution includes a complex of a protein and a predetermined number of substance emitting a fluorescence, or the substance emitting the fluorescence. The solution containing the fluorescent molecules is formed into a fine liquid droplet containing the molecule-group by a ultrasonic wave, and it is preferred that the molecule-group comprises four fluorescent molecules or less (the average number of fluorescent molecules). The substrate, on where the molecule-group absorb, includes a silicon wafer, and may be disk-shaped.

A reason for drying the substrate is that Raman scattering of the solvent which is one cause for a background light is reduced in comparison with that in the solution. Moreover, reactions of the solvent with the molecules which is a cause for deterioration of the fluorescent molecule, such as a dye molecule, can be greatly prevented. Heating and vacuum drying could remove the fluorescent molecule on the substrate.

The device for determining a sequence of base of nucleic acid according to this invention comprises a light source for irradiating a pulsed excitation light to a local area where a base is present, on a flat substrate with the base of the nucleic acid in the sequence, fluorescence detecting means for detecting the fluorescence generated from the base present in the local area on the flat substrate, and identifying a kind of the base, based on a wavelength distribution of the fluorescence, and lifetime of the fluorescence, and moving means for moving the local area on the flat substrate along the sequence of the base.

The fluorescence detecting means may include a photon counting system for determining a location of a base in the local area.

This constitution enables the method for determining a sequence of base of a nucleic acid according to this invention to be realized.

Characteristics of this invention in addition to the above-described first and second characteristics are that the substrate is a disk-shaped silicon wafer. This produces the merit of downsizing the device, and others. Cooling means may be included for increasing fluorescence intensities in detecting fluorescence of the respec-

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tive bases.

The present invention will become more fully understood from the detailed description given hereinbelow and the accompanying drawings which are given by way of illustration only, and thus are not to be considered as limiting the present invention.

Further scope of applicability of the present invention will become apparent from the detailed description given hereinafter. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art form this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagrammatic view of a proposed device for determining a sequence of base of a nucleic acid.

Fig. 2 is a diagrammatic view of the device for determining a location of a molecule-group and a the numbers of a fluorescent molecule in the molecule-group according to this invention.

Fig. 3 is a view of a fine liquid droplet 22 containing the molecule-group to be adsorbed on a silicon wafer as a flat substrate 20.

Fig. 4 is a diagrammatic view of moving means for moving the substrate as shown in Fig. 3.

Fig. 5 is a view of an absorption spectrum of a SA-FLB/B mixed solution.

Fig. 6 is a view explaining that the mixed solution of Fig. 5 does not follow Beer's law.

Fig. 7 is a view of an absorption spectrum of a SA-FLB solution.

Fig. 8 is a view explaining that the mixed solution as shown in Fig. 7 follows Beer's law.

Fig. 9 is a view explaining that the number of FLB/B bonding with SA is estimated by the comparison of the experimental data ($\bullet$) with the standard sample (Δ).

Fig. 10 is a view of fluorescence spectra of respective solutions of (1) FLB alone, (2) SA-FLB/3B, (3) SA-FLB/2B, (4) SA-3FLB/B and (5) SA-4FLB.

Fig. 11 is a view of changes of relative fluorescence quantum yield of the respective solutions as shown in Fig. 10 depending on SA and the number of FLB.

Figs. 12 - 14 are views of distributions of an area on the surface of the substrate occupied by one of liquid droplets containing a fluorescent molecule when their atomizing time is 10 seconds (Fig. 12), 7 seconds (Fig. 13) and 3 seconds (Fig. 14).

Figs. 15 and 16 are views of fluorescence intensity distributions (relationships between the number of fluorescence photons per unit period of time and the appearance frequency of the photons) of SA-FLB/3B mixed solution. The number of intensity is relative quantity, not the number of photons.

Fig. 17 is a view of a fluorescence intensity distribution (relationship between the number of fluorescence photons per unit period of time and the appearance frequency of the photons) of a solution of DSFL alone. The number of intensity is equal to the number of photons in this figure.

Fig. 18 is a view of theoretical values W(N) of a quantized fluorescence intensity distribution and the measured distribution of DFL (shaded) from Fig. 17.

Fig. 19 is a view explaining a relationship between the transition moment of DSFL and the polarization of the 488mm-laser beam.

Fig. 20 is a diagrammatical view of the device for determining a sequence of base of nucleic acid according to this invention.

Fig. 21 is a view explaining the irradiation of a laser beam in the device of Fig. 19 (including the cooling means).

Figs. 22 - 24 are diagrammatic views of specific structures of the cooling means 34 as shown in Fig. 21.

Fig. 25 is a view of applying bases in their sequences on the disc-shaped substrate 20.

Fig. 26 is a view of the substrate 20 with absorbed bases.

Fig. 27 is a detailed structural view of the flow cell 10 in Fig. 25.

Fig. 28 is a diagrammatic view of an example of the device for determining a sequence of bases of nucleic acid according to this invention.

Figs. 29 - 32 are pictorial views of the principle for luminescence detection for identifying a kinds of a base (monoclonal antibodies applied to the substrate 20).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As described above in connection with the prior art, a measurement of locations of fluorescent molecules and the number of the fluorescent molecules has not been so far developed. The above field makes progress

at present. A method for detecting locations the fluorescent molecules and measuring the number of the fluorescent molecules is an important part of the present invention. Firstly, the measuring method conducted by an inventor of the present invention, and the device for the measuring method will be explained.

5 (Determination of Number of fluorescent molecules)

One characteristic of this invention is that for the confirmation of presence of a molecule-group comprising the fluorescent molecule, a excitation light is irradiated to count the number of generated fluorescent photons per unit time as quantized fluorescence intensity in proportion to the number of the fluorescent molecules, 10 while a location (a fluorescent spot) of a molecule-group and the number of a fluorescent molecule in the molecule-group present in a local area irradiated with the excitation light is determined based on an appearance frequency distribution of the quantized fluorescence intensity formed on a two-dimensional image corresponding to the local area.

Fig. 2 is a block diagram of an example of a device for a method for detecting a molecule-group comprising 15 a fluorescent molecule according to the present invention. This device comprises an excitation light source 30 for irradiating the excitation light to the surface of a silicon wafer as a substrate 20 with the molecule-group to be measured absorbed on thereof (this silicon wafer is covered with an insulating natural oxide layer), an optical microscope objective 42 for condensing a fluorescence generated from the fluorescent molecule in the molecule-group, and a photon counting system A (comprising a photon-counting camera 40, camera controller 20 44, computer 45, a monitor 46, and MO disk unit 47) as means for detecting at an excitation light irradiating position the fluorescence from the fluorescent molecule. The optical microscope objective 42 and the photon counting system A are connected by a microscope body 41. The substrate 20 is positioned in a clean booth of Class 1000 or less to be in a clean ambient atmosphere. This device can be used not only for a base of nucleic acid, but also for various substances including protein as long as the substance generates fluores- 25 cence. A molecule which does not generate the fluorescence can be used by being bonded with a predetermined number of substance emitting a fluorescence to be measured by this device.

It is preferable that the excitation light source 30 continuously irradiate intense light, e.g., coherent laser beam, to a part of the substrate 20 on where the molecule-groups comprising fluorescent molecules is adsorbed. Here, a 488 nm-wavelength argon laser beam (Spectra-physics 2030) is adjusted by a calibrated power 30 meter (Spectra-physics 385) to be an average power of 7 - 20 mW. Then, the beam is condensed by a lens 32 with a 50 cm focal length to be applied. The excitation light source 30 is so arranged that excitation light enters slantly α=70° as in Fig. 2, so that the reflected beam on the surface of the substrate 20 reflects slantly.

The flat substrate will be defined here. According to the results of the embodiments (e.g., Fig. 17) which will be explained later, an intensity ratio between a fluorescence intensity and a background light intensity is 35 about 100 : 1 (larger this ratio, the better). In the following embodiments, an irradiation angle is about 20° to the horizontal plane, but the irradiation angle is not limited to the angle as long as the laser beam (excitation light) and the reflected beam are not directly irradiated to an objective (incorporated in the optical microscope 42) for condensing the fluorescence and can illuminate a set local area of the substrate 20.

Then, in the specification "flat" will be defined as follows. That is, the "flat" means a state in which the 40 fluorescence can be measured at an intensity ratio of above 10 : 1 between the fluorescence and a background light when the laser beam is incident on the surface of the substrate 20 at an irradiation angle which meets the above-described conditions.

A background light intensity is evaluated in a photon count value (the number of fluorescent photons per unit time) in an area on a two-dimensional image where a fluorescence spot (which are a pixel group corre- 45 sponding to a position on the substrate 20 where the fluorescence is detected and indicates the location of the molecule-group to be measured) is absent, and which has the same area of the fluorescence spot.

An intensity ratio between the fluorescence and the background light is limited to 10 : 1 or more to limit the average number of fluorescent molecules (called a molecule-group) contained in one liquid droplet to below 4 so as to facilitate the discrimination of an individual number. That is, since a probability of one liquid droplet 50 containing ten fluorescent molecules in a Poisson distribution of the average number of 4 is can not be ignored, even in such case the fluorescence and the background can be discriminated from each other by setting the intensity ratio between the fluorescence and the background light at 10 : 1 or more.

The photon counting system A is a system for detecting the location of the molecule-group adsorbed on the substrate 20, and the number of the fluorescent molecules at the location, and has to be capable of de- 55 tecting a feeble light. In this embodiment, the system A is an imaging/image analyzing system (ARGUS 50 VIM 3, by Hamamatsu Photonics K. K.) with the optical microscope 42 mounted on through the microscope shaft 41, and can two-dimensionally detect the feeble light by two-dimensionally counting the fluorescent photons. The optical microscope 42 for condensing fluorescence from the fluorescent molecules, in this embodi-

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ment, includes an objective (OPTIPHOT XP, by NIKON) with a magnification of 40 (0.55 NA) or 100 (0.75 NA). The 100 x objective; NIKON CF M plan SLWD, NA = 0.75 and working distance (WD) = 4.3 mm. The 100 x objective was used in the experiment using disodium fluorescein (DSFL). The x 40 objective was used for the droplet size determination shown in Figs. 12-14, and used in the experiment using streptoavidine-dye complex.

The operation principle of the photon counting system A is described by T. Hayakawa : Image Analysis in Biology, ed. D. - P. Hader, (CRC Press, Boca Raton, 1992), Chap. 5, pp. 75 -86.

The photon-counting camera 40 has 512 x 512 pixels and high sensitivity (VIM 3), and one pixel has a 0.3 µm-width with the 40-magnification objective and 0.12µm-width with the 100 x objective. The fluorescence from the fluorescent molecules is converted into a image signal by the photon counting camera 40, and the signal is stored (counting the fluorescent photons), image-processed, recorded and image-displayed by a camera controller 44, a personal computer 45, a MO disk unit 47 and a monitor 46.

The substrate 20 is controlled by a drive device (moving means) as shown in Fig. 4 in terms of its position and rotation so that a relative position of a local area of the surface of the substrate to be in a field of the optical microscope 20 and the photon counting camera 40 can be controlled.

In Fig. 4, the drive device horizontally moves an X-Y stage 100 by a first and a second pulse motor 101, 102 in x-direction and y-direction in Fig. 4. A rotary stage 103 fixing the substrate 20 is mounted on the X-Y stage 100. The local area of the substrate 20 can be moved in the x-, the y- and θ-directions. These parameters x, y, θ can be controlled independently to one another by a computer 104, whereby a position where a liquid droplet containing a molecule-group adsorbed on the substrate 20 can be spirally displaced (according to the embodiments which will be explained later).

The above first and the second pulse motors 101, 102 are provided by marketed ones that can control displacements in the x- and y-directions by a minimum unit of 1 µm (MSS-150/200, Microscanning stage, 1993, vol. 1, CHUO PRECISION INDUSTRIAL CO., LTD - CATALOG).

It is possible that wavelength selecting optical components are provided for shutting light other than the fluorescence intrinsic to an object to be measured in the microscope body 41 between the light detecting surface of the photon-counting camera 40 and the optical microscope 20 so as to prohibit incidence of scattered light of the excitation light. Similarly with Fig. 1, the wavelength selecting means comprises a wavelength selecting filter (for example the color filter BA 520 -560, by NIKON), and a dichroic mirror (for example DM 510, by NIKON). The choise of a mirror and a filter depends on the color of fluorescence. In this embodiment, BA520-560 and DM-510 are used because fluorescence spectrum of DSFL has the 520 nm-maximum. The laser beam out side of the microscope can prohibit the incidence of the laser beam to be the background light and scattered light on the surface of the substrate 20. Approximately 60% of the fluorescence photons from DSFL pass through the combination of the filter and the mirror.

Then, the method for determining the location of the molecule-group and the number of the fluorescent molecules in the molecule-group by this device will be explained in the sequence of the steps thereof.

The fluorescent molecules to be measured are solved and diluted in a predetermined solvent. This solution is absorbed on the surface of the substrate 20 in atomized condition (fine liquid droplets) (Fig. 3). Then the substrate 20 is dried in a clean atmosphere to prepare the substrate 20 with the absorbed molecule-groups. The solvent has high purity. One or more than one liquid droplet is in an view area of the microscope substantially corresponding to a resolution of the microscope objective 42 (an average number of fluorescent molecules in one liquid droplet is 4 or less and one molecule-group comprises these fluorescent molecule) (this will be explained later). Drying of the solvent decreases Raman scattering of the solvent being one reason causing the background light, and chemical reactions between the solvent and the fluorescent molecules, which is a cause of degradation of a fluorescent material, is greatly hindered. It is very possible that drying by heating or vacuum drying removes the specimen molecules. In case that a silicon wafer is used as the substrate 20, the fluorescent molecules are fixed by absorption, and the fluorescent molecules cannot be easily dissociated. Accordingly, the substrate 20 can be repeatedly used. The silicon wafer as the substrate 20 is used as supplied, thereof surface being covered with insulating native oxide (SiO_2). Furthermore, the reflectivity of the substrate 20 (40%) can be measured with a Xe lamp coupled with a green interference filter (520nm) and a power meter can be equipped with an integration sphere (UDT S370).

An average number of the fluorescent molecules contained in the liquid droplet 22 is determined by a concentration of the solution containing the fluorescent molecules and the droplet size. Accordingly, when the size of the liquid droplet 22 is assumed constant, the average number of the fluorescent molecules in one liquid droplet 22 can be controlled by changing the concentration of the solution. Actually, the size of the liquid droplet 22 can be arbitrarily set unless the liquid droplet 22 is so larger as to be outside a visual field of the detecting means.

In the embodiments which will be explained later (for example, Figs. 12 - 14), the liquid droplet 22 does not have a constant size, and has distributions (Figs. 12 - 14 are results of the inventor's evaluation of the

distributions). The distributions show that the liquid droplets 22 of volumes of 10 μm^3 or less occupy 90% or more. Since this embodiment can prepare the liquid droplets 22 of a 10 μm^3 or less volume, it is easy to calculate a solution concentration which allows one liquid droplet to have 4 or less as to the average number of the fluorescent molecules. In computation, one liquid droplet can have an average number, 4 by setting the concentration of the solution containing the fluorescent molecules at 0.68 nano mol/liter or less.

A reason for limiting an average number of the fluorescent molecule in the one liquid droplet to 4 is to facilitate the discrimination of the individuals of the fluorescent molecules. That is, when the one liquid droplet having a set volume has 5 or more as to the average number of the fluorescent molecules, it is very difficult to discriminate a Poisson distribution contour, i.e., a contour of a graph plotting probabilities of the number of the fluorescent molecules appearing with respect to the number of the fluorescent molecules. For example, it is easier to discriminate the Poisson distribution contour with one average molecules number from that with two average molecules number than the Poisson distribution contour with 5 average molecules from that with 6 average molecules.

Then, the substrate 20 is set in the device of Fig. 2, and while the laser beam is being irradiated, an area on the substrate 20 emitting light is measured. Since the laser beam is incident and reflected slantly, they do not enter a field of the optical microscope 42, and the background light has a very small intensity. In particular, the substrate 20 is a silicon wafer, the scattered light has a small intensity because of a high speculum degree of the surface thereof. Furthermore, since no solvent molecules are on the surface of the substrate 20, there is little as to the Raman scattering, and the background light has an small area which allows fluorescence from one molecule to be detected. The photon counting system A can measure a fluorescence intensity (the number of the fluorescence photons per unit time) and fluorescent spots where fluorescence of the intensity appears (an area where the fluorescent spots are present) on each screen corresponding to each local area, whereby it can be detected how many and where fluorescent molecules are present.

In the embodiments which will be explained later (e.g., Fig. 2), an irradiation angle of the laser beam to the substrate 20 is set at $\alpha=70°$, and an intensity ratio between the fluorescence and the background light is better than 100 : 1.

This detection of fluorescent molecules will be detailed based on actual measured results.

Position dependence on the light detecting efficiency sometimes occurs because of uneven irradiation of laser beams (TEM_{00} mode) to the substrate 20, and this is measured beforehand. A sheet of opaque glass is placed at a position where the substrate 20 to be set to observe scattering of the laser beam of 488 nm wavelength. Significant parts of 512 x 512 pixels are marked. A brightness difference of the effective parts is about ±5% at most when the 100 x, NA=0.75 objective is used. In this embodiment, the laser-beam diameter on the surface of the substrate 20 is estimated to be 300 μm ($1/e^2$), and the field of the vision in the photon counting system A is approximately a 60 μm in diameter when the 100 x, NA=0.75 objective is used.

Here, the fluorescent molecules to be measured are provided by complexes of protein and fluorescent substance. The fluorescent substance is fluorescein biotin (FLB), and disodium fluorescein (DSFL). FLB and DFL have, in an aqueous solution, a 489 nm absorption peak ($\varepsilon=90,000$ $cm^{-1}M^{-1}$ at pH=8.4) and a fluorescence maximum at 520 nm (fluorescence quantum yield of $\Phi_f = 0.95$ at pH=8.4) which were measured values given by Hitachi 557 and Hitachi 850). The protein is provided by tetrameres of streptavidin (SA) (the molecular weight 4 x 15,000). This SA has high affinity (dissociation constant $k_d=10^{-15}M^{-1}$) with FLB, A part (B) of FLB specifically reacts with SA, and 4 FLB/B molecules can be bonded (avidin-biotin complexes are produced). This reaction can be used to control the number of the fluorescent substance which bonds with SA. By controlling a mixed ratio (mole ratio) of FLB/B, the number of the FLB molecules which bond with SA can be made 1 - 4.

At first, the solution containing the above-described fluorescent substance to be measured is atomized to be adsorbed in the liquid droplet 22 to the substrate 20 (Fig. 3), and the substrate 20 is dried. The substrate 20 is thus prepared. In this embodiment, a ultlasonic humidifier (Sharp HV-A 200) was used to atomize the sample solution into fine liquid droplets 22, and the drying was conducted in a clean ambient atmosphere. The drying of the solvent reduces the Raman scattering of the solvent which is one cause of the background light, in comparison with that of the solution, with a result that the reaction of the solvent with the fluorescent molecules, which is a cause of degradation of the fluorescent substance, can be drastically prohibited. It is very possible that the heating and vacuum-drying will remove the fluorescent molecules (the molecule-groups) to be measured.

As seen in the embodiments which will be explained later (e.g., Fig. 17), a distribution of sizes (volume) of the liquid droplets 22 has 10 μm^3 or less by 90% or more, and at the maximum number of occurrence in the size distribution the volume is in a range of 3 - 4 μm^3. In this embodiment, as described above, the fine liquid droplets 22 are produced by means of the ultrasonic humidifier, but other liquid droplet generators are described in, e.g., Kin C. N, "Digital Chemical Analysis of Dilute Microdroplets", Anal. Chem., 1992, 64, 2914-2919.

In this embodiment (Fig. 3), a volume for each of the liquid droplet 22 is calculated on the conditions that the liquid droplet 22 is hemispherical and has a 90° contact angle. It is assumed that a fluorescent area (a fluorescent spot) after drying is in an equatorial plane of a hemispherical liquid droplet.

The above-described solution is diluted with super-pure water (Mili-Q water, Milipore). The solution can have a nano-mol /liter concentration, and as will be explained later, the average number of the fluorescent molecules in the one liquid droplet containing one molecule-group can be calculated. Some specimen aqueous solutions were repeatedly atomized onto silicon wafers (the substrate 20) and dried, and measured at 296 K. The aqueous solutions atomized onto the silicon wafers were prepared as follows.

SA was solved in a mixed aqueous solution (pH 7.5) of 10 mM of phosphate and 0.15 M of NaCl (2 mg/ml), and FLB and B were solved in the same buffer solution (phosphate and NaCl). Four kinds of mol ratios of FLB to B, 4:0, 3:1, 2:2, and 1:3, were prepared. A molar ratio which enabled FLB and SA to sufficiently react with each other was 20:1. The four kinds of FLB/B solutions were mixed in 100 µl of SA solution. Then, the above-described buffer solution was added to prepare a 200 µl solution.

After the thus-prepared solution was let to stand at 277 K for 4 hours, the SA-FLB/B mixed solution was passed through a gel filtering column (Superose 12, Pharmacia, bead size: 10 - 11 µm, column size: 1.2 x 30 cm, number: 12) to be separated into sufficiently reacted SA, and unreacted or insufficiently reacted SA. a solution of 10 mM of ammonium carbonate or phosphate (containing no NaCl) was used as a developer liquid (mobile phase). The measurement was conducted on the prepared specimen (the substrate 20 with the above-described fluorescent substance applied to) in next two days since the lifetime of the SA/B complex is about 2.9 days.

The DFL (Exinton) is used to estimate a size of liquid droplets to be atomized. A 2.3 x 10^{-5} M-DFL solution is atomized onto the silicon wafer (the substrate 20), and the substrate was mounted on the device of Fig. 2. While the laser beam was being irradiated, a size of an area (pixel^2) where fluorescence was generated was measured by the photon counting system A. It should be noted that the sensitivity of the detector was lower than that used in the photon counting system A, the light source was a halogen lamp, and one pixel was 0.3 µm in the droplet size measurements. On the assumption that a contact angle of the liquid droplet was 90°, the size of the area is converted into a volume of the semispherical liquid droplet 22a (Fig. 3).

These fluorescent molecules, FLB+SA and DSFL were used in consideration of biological and photophysical applications. The measurement of the first substances to be measured (SA, FLB, B) are for applications to immunoassay and DNA sequence. The measurement of the second substance to be measured (DFL) is for applications to photophysical studies.

The technique of forming a SA-FLB complex is based on a stoichiometric complicated composition of SA and FLB/B. The number of SA molecules bonded at four sites is measured by computing a ratio of light absorptions (A(489), A(280)) with respect to 489 nm wavelength and 280 nm-wavelength. Light absorption at 489 nm is only by FLB, and light absorption at 280 nm is only by FLB and B. FLB (d-biotin) does not absorb light in a 200 - 900 nm range. But Beer's law is not satisfied in a state where both SA and FLB are present (3 - 12 µM in 10 mM triethanolamine/HCl, pH=8.42).

Fig. 5 shows an absorption spectrum of FLB/B mixed with SA. The spectrum is for a compounds of FLB/B and SA having a ratio of 4 : 1. The spectrum was measured without column chromatography. In addition to the fact that the spectrum does not agree with Beer's law, as shown in Fig. 6, the half-value width of 489 nm increases with the increase of the FLB.

In Fig. 5, the measurement was conducted on a mixture of FLB/B and SA solved in a 10 mM triethanol amine/HCl at 296 K. A mol ratio between the FLB/B and the SA is 4 : 1. In this range, d-biotin does not absorb light. The light absorption near 280 nm is mainly due to tryptophan in the SA. In Fig. 6, black plots mean that A (280) does not linearly increase with increase in a concentration of the FLB. White plots mean that a half-value width at a 489 nm peak widens with increase of FLB concentration.

Figs. 7 and 8 show that a complex with only FLB follow Beer's law, and no expansion of a half-width value takes place in a same concentration range as in Figs. 5 and 6. The number of the FLB bonded with SA can be determined by referring to a ratio between A(489) and A(280) in which the FLB has small contribution. The FLB/B bonding causes a red shift (shift to a shorter wavelength) of tryptophan near 289 nm. Based on the above, on the assumption that the FLB/B and the SA in a mixed solution are completely bonded with each other, a ratio between A(489) and A(280) is given in Fig. 9, and, based on the ratio, the number of the FLB can be estimated.

To stoichiometrically react SA with FLB/B, a reaction time is important. Cause for this is not known. The reaction solution was let to stand overnight or more, and the number of the FLB/B bonded to the SA decreased. A ratio between A(493) and A(280) depends on the fraction number of chromatography. Black marks in Fig. 9 indicate fractions with maximum ratios between A(493) and A(280), and these fractions were used. Ratios of 4FLB, 3FLB/B, 2FLB/2B and FLB/3B considerably well agree with a ratio, as a standard value, in the case

that FLB/B and SA are mixed exactly in a mol ratio of 4 : 1. But a ratio of 3FLB/B apparently disagrees with the ratio as the standard value, and different ratios were obtained every time the above-described solution was prepared. A possible reason for this is that the FLB/B separates from the SA in chromatography.

Fig. 9 shows that the number of FLB/B bonded with SA can be calculated based on an absorption A(489) at 489 nm, and an absorption A(280) at 280 nm. Only FLB contributes to A(489), and A(280) includes contribution of SA and FLB. Estimation of A(489)/A(280) was conducted at various ratios between the SA and the FLB/B. Black plots indicate that FLB/B-SA mixture from chromatography was used, and white triangular plots indicate that mixing ratios of the SA and the FLB/B was exactly 1 : 4. The latter was used as a standard value for determining the number of the FLB bonded with the SA.

It is known that fluorescent molecules at high concentraion in water do not agree especially with Beer's law. According to some past studies as to an organic dye, it is found that the absorption spectrum varies much depending on the concentration. Foster and Konig report that it is found based on changes of absorption spectra of some organic dye molecules (fluorescent molecules) containing a fluorescent substance in water that the organic dye molecules form dimers at high concentration (10^{-3} - 10^{-1}M). Koizumi and Mataga report change of absorption spectrum in an aqueous solution containing fluorescent molecules in the presence of electrolytic polymers. Based on these studies, it is considered that changes of the spectra are caused by cohesion effect of the fluorescent molecules to the polymers. Considering these past studies, and the bonding between SA and B due to their high affinity, it is considered that similarly sufficient local adhesion effect of FLB to SA can be present. This is also an optical evidence of interaction between SA and FLB. For example, bovine serum albumin, which has no affinity with FLB, causes no changes to FLB spectrum.

Then, a relative fluorescence quantum generation ratio of FLB bonded with SA, and a fluorescence spectrum were measured. Taking into consideration abnormal behaviors of the absorption spectra, it is considered that something takes place in the fluorescence characteristic of FLB upon bonding with SA. Fig. 10 shows fluorescence spectra of (1) FLB alone, (2) FLB/3B bonded with SA, (3) 2FLB/2B bonded with SA, (4) 3FLB/B bonded with SA, and (5) 4FLB bonded with SA (the bonding with SA was caused by solving with a 10 mM triethanol amine/HCl at pH8.42 and 296 K). The contours of the spectra are the same, and the peaks are not shifted. But when the FLB first bonded with the SA, the intensity extremely dropped and considerably decreased as the FLB went on bonding. Fig. 11 shows results of calculation of relative fluorescence quantum generation ratios of (1) to (5) attributed to SA. Fluorescence due to quenching of the SA shows that there are extinction reactions between the amino acid of SA and FLB. The mechanism of fluorescence due to the quenching depending on the number of FLB is considered the same as the self-quenching (which was reported in the above-described past study) by aqueous solutions containing fluorescent molecules.

Considering the above-described invention, a selection of suitable fluorescent molecules was conducted based on the results of Figs. 10 and 11. Since a fluorescence quantum generating ratio and a fluorescence coefficient decrease with the number of the FLB, only SA-FLB/B (monomer) can be measured for confirming a detection of the number of the molecules. In view of this, monomers are used in detecting the number of the fluorescent molecules.

According to this invention, it is necessary that one liquid droplet contain a small number of fluorescent molecules as a molecule-group. Since the number of the fluorescent molecules follows Poisson distribution in the liquid droplet, the number of the fluorescent molecules in the liquid droplet can be calculated with the Poisson distribution. The inventors prepared a highly purified solution containing DFL, caused the DFL to adsorb on a silicon wafer in fine liquid droplets by a ultrasonic humidifier to measure the droplet size (volume), and the number of the fluorescent molecules in the one liquid droplet. The respective holizontal axis in Figs. 12 to 14 show distributions on areas on the silicon wafer 20 occupied by one liquid droplet for a 10-second, 7-second and 3-second periods of atomizing time. Frequencies (the number of liquid droplets) for sizes of areas (in $pixel^2$ unit, 1 pixel=0.3 μm) are shown. Positions of the peaks are a little shifted as the atomizing period of time is longer, but actually contours of the distributions, and the positions of the peaks are independent of the atomizing periods of time. Since a maximum peak is about 30 - 40 $pixel^2$ (3 - 4 $μm^3$), the number of fluorescent molecules in one liquid droplet is 1.00 - 1.56 for 1.0 nm/l concentration of the solution.

Liquid droplets each with a very small number of molecules as a molecule-group thus contained are caused to stick to the substrate 20 (silicon wafer), and then 100 or more light emitting areas on the substrate 20 were measured by the device of Fig. 2. Their fluorescence intensities, and frequencies of fluorescence spots of the intensities appearing were plotted.

Actually, the number of fluorescence photons per unit period of time, which were generated from a local area on the substrate 20 with excitation light irradiated was used as a quantized fluorescence intensity. A distribution of a frequency of appearance of the fluorescence photons is formed on a part of a two-dimensional image corresponding to the local area on the substrate 20. An area with a higher frequency of appearance of the fluorescence photons in the distribution of frequencies of appearance of the fluorescence photons is a

fluorescence spot corresponding to the molecule-group in the local area. Based on a location of the spot, a location of the molecule-group (four molecules or less) can be identified.

Since the fluorescence intensity has a specific value corresponding to the number of fluorescent molecules, the number of fluorescence photons is counted by the above-described photon counting system A to measure, based on the counted value, the number of fluorescent molecules in one fluorescent spot (2-D photon counting method). The 2-D photon couting method is described by T. Hayakawa : Image Analysis in Biology, ed. D. - P. Hader, (CRC Press, Boca Raton, 1992) Chap. 5, pp. 75 - 86.

Accordingly the above-described fluorescence intensity is a absolute value based on the 2-D photon counting method. In Fig. 17 which will be explained later, the horizontal axis is "the number of photons". In Figs. 15 and 16, however, the horizontal axis is relative photon number. The results of Figs. 15 to 17 have a 60-second data accumulating period of time. An excitation light intensity is 7 mW in Figs. 15 and 16 and 20 mW in Fig. 17. A polarization direction of the laser beams in Figs. 15 to 17 is parallel with the sheets of the drawings as shown in Fig. 19.

Figs. 15 and 16 show the results of the measurement using FLB/3B bonded with SA on the entire surface of the substrate 20. (The optical microscope 42 was 40x, 0.55 NA.) Some peaks appear at every 500, which means that an fluorescence intensity is quantized, and this measurement detects the number of fluorescence spots having from 1 to 4 sample molecules. Intensity smaller than 500 on the horizontal axis might be attributed to scattered light of 488 nm wavelength due to dust particules on the silicon wafer. A minimum peak value clearly appears at 500 on the horizontal axis in Fig. 16. This peak is considered to be attributable to the single SA bonded with the FLB/3B. Fig. 15 and Fig. 16 have different buffer liquids from each other in column chromatography. In Fig. 15, phosphate (pH=7.5) was used alone, and in Fig. 16, ammonium carbonate was added (pH=7.5). These differences are considered to result from that optical noises due to bases of the buffer liquid detected on the surface of the substrate 20 because of volatility of the ammonium carbonate are suppressed.

Fig. 17 shows the result of the measurement using DFL alone. (In this case, the optical microscope 42 was 100 x, 0.75 NA). In this case as well, some peaks appear at every 1,000, and fluorescence intensities are quantized. The experiment in which water alone was atomized onto the silicon wafer 20 shows that the fluorescence intensities at 800 or less might be due to dust in the water. It can be affirmed that the fluorescence intensity at 1,000 is of the single molecule of the DFL.

The frequency of the quantized fluorescence intensities can be calculated based on distributions of sizes of liquid droplets of Figs. 12 to 14 and the concentration of the sample solution, and can be compared with the experimental results in Fig. 17. The number of fluorescent molecules in one liquid droplet follows Poisson distribution (Formula 1).

$$W(N) = \mu^N e^{-\mu}/N! \quad (1)$$

In Formula 1, W(N) represents probability that N molecules are contained in one liquid droplet, and μ indicates the average number of molecule in one liquid droplet. Since some distribution occur in a size of liquid droplets upon atomization, the actual number of them is expressed by the following Poisson's equation with a weight of the distribution of liquid droplet sizes.

$$W(N) = \sum_{\mu} f(\mu)\mu^N e^{-\mu}/N! \quad \cdots \quad (2)$$

$$\sum_{\mu} f(\mu) = 1 \quad \cdots \quad (3)$$

In Formulas 1 and 2, $f(\mu)$ represents a rated weight coefficient and is calculated based on the respective size distributions of liquid droplets of Figs. 12 to 14. Fig. 18 shows the calculation results of the W(N) together with the measured results (shaded) of DFL (both data are normalized at frequency of N=1). These results show good agreement, which means that the number of fluorescent molecules in each fluorescent spot can be detected.

Next, the data of Fig. 17 obtained by using the suitable fluorescent molecules DSFL for theoretical studies will be quantitatively compared with the number of observable fluorescence photon by using parameters of excitation light intensities, fluorescent molecule light absorption and emission, fluorescence measuring detectors or optical components, and others.

The above-described "Proc. Natl. Acad. Sci. USA 86 (1989) 4087-91" contains a relevant description in connection with this evaluation method.

An excitation light (wavelength: 488 nm, output: 20 mW) is irradiated to the surface of a silicon wafer 20

in a 300 μm diameter (it is defined that the laser beams have a Gaussian space intensity distribution and a diameter which yields a $1/e^2$ intensity). An excitation light intensity (I_{ex}) is given by

$$I_{ex} = 2.37 \times 10^{19} (\text{photons/sec/cm}^2)$$

A fluorescence photon number (I_{abs}) emitted by one fluorescent molecule per second is given as follows when a molecular light absorption coefficient is represented by ε.

$$I_{abs} = 3.8 \times 10^{-21} \cdot \varepsilon \cdot I_{ex}$$

$$= 3.8 \times 10^{-21} \times 9 \times 10^4 \times 2.37 \times 10^{19}$$

$$= 8.105 \times 10^3 (\text{photons/sec/molecule})$$

where a molecular light absorption coefficient ε at a disodium fluorescein (DSFL) excitation light wavelength (488 nm) was 9×10^4/cm/M.

A fluorescence intensity (I_f) is given by multiplying this value I_{abs} by the fluorescence quantum yield. That is, since the fluorescence quantum yield of disodium fluorescein is 0.95, the fluorescence intensity I_f is given as follows.

$$I_f = I_{abs} \times 0.95 = 7.69 \times 10^3 (\text{photons/sec/molecule})$$

Furthermore, when a data accumulating time is 60 seconds, the total number of the fluorescence photons (I_{tot}) generated from one fluorescent molecule is given as follows.

$$I_{tot} = I_f \times 60 (\text{sec}) = 4.61 \times 10^5 (\text{photons/molecule})$$

On the other hand, efficiency of detecting fluorescence photons is evaluated using known parameters as follows.

$$I_{abs} = 4.61 \times 10^5 \times 0.12 \times 0.563 \times$$

$$0.08 \times 0.60 \times 0.73 \times 1.4$$

$$= 1.53 \times 10^3 (\text{photons/molecule})$$

where a quantum yield of the photocathode of the photon counting camera is 0.12 at the fluorescence maximum (520nm), and the upper limit of condensation efficiency of the objective (x 100, 0.75 NA) is 0.56 (=0.75^2), quantum efficiency (η_1=12%) around 520 nm, available transparency (T_1=8%)[21] and the maximum fluorescence collection efficiency (η_2=NA^2=56.3%) of the objective, the transparency of the DSFL fluorescence photons through the band-path filter and the dichroic mirror (T_2=60%), the enhancement of the fluorescence collection by the silicon wefers (η_3=140%), and the total transparency (T_3=73%) of other optics between the objective and the photocathode in the photon-counting apparatus. Note that the surface of the silicon wafers are as smooth as a mirror, thereby improving fluorescence collection efficiency.

In Fig. 17, a minimum unit of the quantized fluorescence intensity is 10^3 (photons), and this is considered to be the number of the fluorescence photons which can be measured from one fluorescent molecule. Thus, the number of the actually observed photons 10^3 is consistent with the calculated result ($I_{abs} = 1.53 \times 10^3$ photons/molecule).

In the case that a polarization direction of laser beam as the excitation light is parallel with the sheet of the drawing (X-Z surface) as shown in Fig. 19, a direction of transition moment of a fluorescent molecule to be measured is important.

When a direction of the fluorescent molecules are fixed on the substrate 20, light excitation efficiency, in the case that the fluorescent molecules are excited by light with a set polarization direction, depends on transition moment of the fluorescent molecules, and an angle of the excitation light to the polarization direction. That is, when both are parallel with each other, the light excitation efficiency is maximum, and no excitation takes place when both are normal to each other.

When both form different angles for respective fluorescent molecules, the quantized fluorescence intensities (the number of fluorescence photons) in Fig. 17 will not be obtained. To understand the results of Fig. 17, it is necessary to consider that the respective fluorescent molecules are moving.

"PHYSICAL REVIEW LETTERS, Vol. 48, No. 7, pp. 478-481, 1982" and "CHEMICAL PHYSICS LETTERS, Vol. 114, No. 1, pp. 103-108, 1985" disclose that the dye (fluorescein) which has produced the results of Fig. 17 has transition moment makes a procession, as shown in Fig. 19, at 53±2° to the vertical line with respect to the surface of the substrate 20. In this state, since the vertically polarized excitation light interacts with a

z-axis component of the transition moment, constant excitation efficiency can be obtained with respect to all the fluorescent molecules.

It is understood from the above that a fluorescence intensity proportional to the number of fluorescent molecules can be observed. In other words, fluorescence photons are counted, whereby, based on a count value, the number of fluorescent molecules in one fluorescent spot corresponding to one molecule-group can be seen.

Thus the measurement of the number of fluorescent molecules on two kinds of fluorescent molecules connected a protein with fluorescent substances, and diluted fluorescent molecules was successful.

In the prior art, the background light has such large that fluorescence from respective fluorescent molecules are buried in the background light and cannot be detected. But this invention has succeeded in making the detection possible and is a great breakthrough. This invention is applicable to various analyses. This invention will make great progress in automation of immunoassay, chromatography and DNA analysis, and will enable super-high speed fluorescence analysis.

As described above, a single molecule can be detected by causing fluorescent molecules adsorbed on the surfaces of solid substrates at room temperature, and detecting locations and the number of fluorescent molecules by two-dimensional photon counting method. Locations of single molecules are detected by means of a conventional optical microscope, based on fluorescent spots at a limit of its resolution. Fluorescent spots are scattered in a field of the microscope, and the number of the molecules in a fluorescent spot is a very important factor in detecting single molecules, depending on intensities of fluorescence from the fluorescent spot. According to the above-described method, one molecule is present in one fluorescent spot, whereby the detection of single molecules is enabled.

In the conventional wet-type detection in which fluorescent molecules to be measured are solved in a solvent, Raman scattering in the solvent (water or others) causes background noises, which makes the measurement very difficult. According to the method of this invention, single molecules can be well detected by selecting a suitable substrate, It is very convenient and effective to use ultrasonic wave (created from ultrasonic humidifiers or others) on the substrate. Salts deposited from buffer liquids are sometimes present on the substrate together with fluorescent molecules to be measured, and sometimes determine levels of background noises. Buffer liquids are not always necessary to prepare a specimen solution depending on kinds of fluorescent molecules, but a buffer liquid has to be carefully selected.

Furthermore, fluorescent molecules to be measured adsorbed on the substrate well, and the substrate can be used in other measurements and is usable in the measurement by, e.g., STM and ATM, which are microscopes with good space resolution. It is possible to use STM or ATM to confirm presence of single molecules, and use fluorescence from fluorescent molecules as means for discriminating kinds of the fluorescent molecules (it is needless to say that the detection method of this invention, which uses fluorescence, can discriminate kinds of molecules). The method of this invention can be one method for determining sequences of bases constituting a single fragment of nucleic acid. That is, the bases of DNA are cut off one by one from the end, using exonuclease III , and the bases are discriminated one by one. In this case, the base are placed on predetermined positions on the substrate which are determined by a cut sequence.

U.S. Patent No. 4,962,037 discloses that the base could be cut off sequentially from the end of a single fragment of nucleic acids by using a solution containing the above-described enzyme, exonuclease III.

(Measurement of a sequence of bases constituting a single fragment of nucleic acid)

Fig. 20 shows a block diagram of one example of the device used in the method for determining a sequence of bases of nucleic acid. This device comprises an excitation light source 30 for irradiating excitation light to a fine area on the surface of a substrate 20, and fluorescence detecting means for detecting fluorescence from bases at the irradiation position of the excitation light and identifying kinds of the bases. The substrate 20 has a very high speculum degree, e.g., a silicon wafer. Nucleic acid of bases as fluorescent molecules are caused to adsorbe on the surface of the substrate by the above-described method. The substrate 20 is placed in a clean booth of class 1000 or less to be placed in a clean atmospheric air or inert gas (for example, Nz, Ar, etc. and an arbitrary complex gas of these elements).

This device may include the above-described photon counting system A to identify locations of the respective bases. Means for moving a local area (area for excitation light to be irradiated to) on the substrate 20 may be the above-described moving means (Fig. 4)

In Fig. 20, it is preferred that the excitation light source 30 can irradiate excitation light to a position on the substrate where bases are adsorbed and is, e.g., a laser beam since coherent laser beams are preferred. When the laser beam source has a wavelength of 300 nm or less, a pulse laser can be used. Excitation light (laser beams) are condensed by a lens 32. The excitation light source 30 is arranged in such a manner that

the excitation light enters slantly as shown in Fig. 21, and reflected beams on the substrate 20 slantly propagate.

A photon counting system A may be included to identify locations of bases in a local area. In this case, as shown in the above Fig. 2, the system A is provided by an imaging/image synthesizing system (ARGUS 50 VIM 3) by Hamamatsu Photonics K.K. with an optical microscope 20 mounted on. The system A can two-dimensionally count fluorescent photons and perform two-dimensional photodetection, whereby locations of the bases can be identified. The optical microscope 20 is for condensing fluorescence from the bases on the substrate 20 and has high magnifications (variable magnification). A photon-counting camera 40 is connected to an imaging/image analyzing system, so that signals are accumulated (fluorescence photon counting), images are processed, records are reserved, and images are displayed, whereby a presence of respective bases can be detected.

A half mirror 56 is for branching fluorescence from the bases on the substrate 20, and branches a larger light amount to a polychromator 52. The polychromator 52 measures fluorescence spectra, and a fluorescence lifetime measuring device 54 measures lifetime of fluorescence. They are provided by an photomultiplier or a streak camera. These members identify kinds of the bases. In place of the half mirror 56, these members, and the photon-counting camera 40 may be mechanically or optically changed over.

The substrate 20 is controlled by the drive means as shown Fig. 4 in its position and rotation so that an area on the substrate 20 to be in a field of the optical microscope 20 and the photon counting camera 40 is controlled. That is, as shown in Fig. 21, the substrate 20 is run at a constant speed in the direction indicated by B. A part of the substrate 20 near an area to be irradiated with excitation light is cooled by cooling means 34.

Fluorescence (autofluorescence) intrinsic to the kinds of the base at room temperatures is so feeble that it is necessary to multiply intensities of fluorescence of all the bases.

For example, "Photochemistry Photobiology, vol. 7, pp. 189-201, 1968" reports it was confirmed that increase of autofluorescence of base A (adenine) can be obtained by lowering temperatures. "Photochemistry Photobiology, vol. 7, pp. 597-612, 1968" reports it was confirmed that increase of autofluorescence of all the bases can be obtained by lowering temperatures.

The cooling means is specifically provided by the cooling end 34a of a cryostat led to the underside of the substrate 20 (e.g., copper block) as shown in Fig. 22, burying balls 34b with high heat conductance between the cooling end 34a and the substrate 20 as shown in Fig. 23, blowing cooling gas (e.g., helium gas) 34d through a pipe 34c as shown in Fig. 24. This cooling has an effect that bases themselves constituting a single fragment of nucleic acid have increased fluorescence as temperatures lower, and dye deterioration is suppressed.

Then the method for determining a sequence of bases of nucleic acid using this device will be explained with reference to the procedures thereof.

First, as shown in Fig. 25, liquid droplet 24 containing base separated from a nucleic acid (e.g., DNA) are dropped onto the substrate to prepare the substrate 20 with the bases adsorbed on (Fig. 26)

The substrate 20 is moved by the moving means as shown in Fig. 4. The substrate 20 is horizontally rotated so that the bases are circumferentially moved to be cut off radially inward and fall liquid droplets 24 containing the bases. In this invention the substrate 20 is a disk, which produces the effect of downsizing the device, and other effects.

It is necessary that sizes of the liquid droplets be as uniform as possible. For example, "Anal. Chem., 1992, 64, 2914-2919" describes a liquid drop generating device which can form substantially 10 μm-diameter liquid droplets.

The flow cell 10 specifically has the structure diagrammatical shown in Fig. 27 and includes 12 supersonic traps 12 in the main body 11. Each supersonic trap 12 has a piezo-element 13 and is connected to a piezo-drive circuit by a line 14. Liquid containing separated bases of DNA is caused to flow through a flow passage 15 of the flow cell 10, and the piezo-element 13 forms standing waves which separate and drop the liquids as shown in Fig. 27. On the other hand, the substrate 20 is rotated at a constant speed by the drive mechanism of Fig. 4 so that a position for a liquid droplet to be fallen onto comes at the center (Fig. 25). Dropped bases stick to the substrate on run in the direction of the arrow C at a substantially constant interval so that one liquid droplet comes into a field of the optical microscope 20.

Nucleic acid is cut off sequentially from the end of a single fragment using a solution containing an enzyme, exonuclease III as described in the immediately above-mentioned reference. The nucleic acid kept in good state at about 37 °C can be cut off at a speed of about 100 pieces/s using the above-described enzyme, and the liquid droplets in fine particles are dropped from the flow cell 10 at a speed of about 300 pieces/s. The frequency of the standing waves are adjusted in such a manner that the fine particles dropped from the flow cell 10 have an about 30 μm diameter, and taking account of Poisson's equation, one liquid droplet contains

one base. Thus the substrate 20 with a single base spirally one after another stuck to can be prepared.

Idealistically, conditions which lowers the possibility of one liquid droplet containing two or more bases are preferred (e.g., a preferred average number is 0.1 or less).

According to this invention, liquid droplets 24 containing bases, and liquid droplets 24 not containing the bases are present on the substrate 20, which is the best method for perfectly recognizing a sequence of the bases.

Then, the substrate 20 with the liquid droplets 24 stuck to is dried to remove the solvent. Accordingly the substrate 20 has solvent molecules which cannot be removed by drying, and the bases stuck to. Since it is very possible that removal of the solvent molecules (heating or vacuum drying) will remove fluorescent molecules (bases) from the substrate 20, the solvent molecules are left on the substrate 20. Drying is effective to decrease Raman scattering from the solvent, and suppress dye deterioration. The air-drying may be conducted together with cooling. The substrate 20 of silicon can be a good specimen. This will be because dangling bonds actively bonded.

The substrate 20 with the bases thus stuck to is mounted on the device of Fig. 20, and the moving means of as shown in Fig. 4 is driven to bring a visual field of the optical microscope 42 near a position where a first one of the liquid droplets to be measured is present. While laser beams are being irradiated, fluorescence from the bases is detected by the fluorescence photon counting system A, and a first one of the bases on the substrate 20 is observed to confirm that the base is within the visual field of the optical microscope 42. It is also confirmed that the base is one field piece. When a plurality of bases are present in the visual of the optical microscope 42, the visual field of the optical microscope 42 is displaced, or a magnification is selected so that one base is present in the visual field. Pulsed laser beams are irradiated from the excitation light source 30 to the base to be measured to excite the same, and a fluorescence wavelength and a fluorescence lifetime are measured by the polychrometer 52 and the fluorescence lifetime measuring device 54. Fluorescence from one molecule enter in visual fields of these members.

The ground that kinds of respective bases can be identified based on fluorescence wavelengths and fluorescence lifetime is disclosed in U.S. Patent Application No. 07/968,868 or European Laid-Open No. 0 556 509 A2.

On the surface of the substrate 20 on which flurescent moleclues adsorb, Raman scattering is extremely little, and the background light has a small area. The laser beams from the excitation light source 30 are prohibited from entering the visual field of the optical microscope 42. The substrate 20 is provided by one (silicon wafer) of high speculum degree. Accordingly the background light has a small area. The background light has smaller areas especially in the reflected case rather than in the transmitted case. As a result, although fluorescence from the respective bases is very feeble, the fluorescence can be measured without being hidden by the background light. Since it is almost impossible that the excitation light may be injected into the fluorescence detecting means 40 by reflecting thereof, the excitation light does not become noise component even when the polychrometer 52 has imperfect cut-off characteristics.

Based on differences of the thus-obtained fluorescence wavelengths and fluorescence lifetimes, the respective bases can be discriminated into A, T, G and C, and a nucleotide in the field of the optical microscope 42 can be identified as A, T, G or C. The fluorescence detection may be conducted off-line to elongate a detection period. Then an irradiation area of the laser beams, i.e., the visual field of the optical microscope 42 is moved by the moving means as shown in Fig. 4 to a next base stuck to the substrate to similarly identify the kind of the base. The observation of the base one after another, and a sequence of the base of a single fragment can be determined.

According to this invention, bases to be measured are stuck to the substrate, and while positional information of the respective bases are obtained, the kinds of the bases are identified. The measurement can be not only excuted repeatedly but also used as a recording medium of nucleic acid.

In addition, the above-described embodiments can have variations.

For example, the substrate 20 is preferably a silicon wafer, but other semiconductors or insulators, or metals depending on cases may be used as long as substantially the same high flatness and speculum are available. But aluminum and gold are vapor deposited films but have disadvantages that they are easily oxidized, and are vulnerable to damages. Metal substrates coated with silicon oxides can be used as the substrates 20 if they have high speculum degrees.

The optical microscope 42, the polychrometer 52 and the fluorescence lifetime measuring device 54 may be arranged as in Fig. 28. In this arrangement, a reflecting mirror 25 reflects fluorescence from fluorescent molecules (bases, etc.) to the polychrometer 52 and the fluorescence lifetime measuring device 54, whereby wavelengths and fluorescence lifetimes can be well measured.

A plurality of photon counting cameras 40 may be provided in the photon counting system A. When one detection is a failure, the detection may be repeated. This is because bases which have been once stuck to

the substrate 20 do not change their positions. Light emission or fluorescence detection can be repeated.

There are light emitting reagents by bonding with bases A, T, G and C. It can be considered to coat the antibodies with these reagents to augment detection of the bases.

Monoclonal antibodies A, T, G and C, which comprise homogeneous molecules, have the characteristic of bonding specifically with base A to C. These monoclonal antibodies are beforehand applied to the substrate 20 (Fig. 29). Bases of nucleic acid are dropped by the flow cell 10 to bond the bases A to C with their corresponding antibodies A to C. The luminescent antibodies A to C have respective luminescent enzymes as shown in Fig. 30 and, in addition, the characteristics of specifically bonding with the bases A to C. The luminescent monoclonal antibodies A to C are dropped, and their surpluses are rinsed off. As a result, as shown in Fig. 31, a monoclonal antibody A on the substrate 20 bonds specifically with the base A, and further a luminescent antibody A on the substrate 20 specifically bonds with the base A. As shown in Fig. 32, a monoclonal antibody T on the substrate 20 bonds with the base T, and further a luminescent antibody T bonds with the base T. Thus, by varying wavelengths of the respective enzymes, it is possible to discriminate the bases by detection of the luminescence.

As described above, according to the method for determining a location of a molecule-group and the number of a fluorescence molecule in the molecule-group, the background light can be suppressed sufficiently enough to directly detect fluorescence from a single fluorescent molecule (a base, etc.) to be measured. Accordingly the quantized fluorescence can be observed, whereby states (location and number) of the molecule (or the molecule-group comprising some molecules) sticking to the surface of the substrate can be detected.

According to the method and the device for sequencing bases of nucleic acid of this invention, fluorescence wavelengths and lifetimes of fluorescent molecules containing the bases on the surface of the substrate are measured after it is confirmed that the fluorescent molecules each is in single molecule state. Accordingly beses contained in the single molecules can be correctly identified, and the bases are identified one after another, whereby a sequence of the bases can be determined at high speed and correctly.

From the invention thus described, it will be obvious that the invention may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

Claims

1. A method for determining a location of a molecule-group and the number of a fluorescent molecule in the molecule-group comprising:

   a first step of irradiating excitation light to a local area on a flat substrate with a molecule-group comprising adsorbed fluorescent molecules;

   a second step of measuring the number of a fluorescence photon per unit period of time, obtained by irradiating said excitation light to said local area on said substrate, as a quantized fluorescence intensity of said molecule-group; and

   a third step of determining a location of said molecule-group and the number of a fluorescent molecule in said molecule-group, based on a measuring position in said local area and the number of said a fluorescence photon from said molecule-group.

2. A method according to claim 1, wherein

   said fluorescent molecule in said molecule-group includes a complex of a protein and a predetermined number of substance emitting a fluorescence, or said substance emitting said fluorescence.

3. A method according to claim 2, wherein

   said excitation light includes a laser beam.

4. A method according to claim 1, wherein

   an irradiation angle of said excitation light to said flat substrate is set so as to avoid an angle which a reflected light from said irradiated excitation light effects on the detecting said fluorescence photon in said step 2.

5. A method according to claim 4,

   said second step further comprising a step of forming an appearance frequency distribution of said fluorescence photon for said unit period of time by plotting a pixel corresponding to a measuring position

of said fluorescence photon on a two-dimensional image corresponding to said local area on said substrate, every time said fluorescence photon is detected by irradiating said excitation light.

6. A method according to claim 5,

said third step further comprising a step of identifying a fluorescent spot where said molecule-group is present, based on said appearance frequency distribution of said fluorescence photon per said unit period of time displayed on said two-dimensional image, and determining the number of said fluorescent molecule in said molecule-group, based on the number of said fluorescence photon measured in said fluorescent spot.

7. A method for forming a specimen with a molecule-group stuck to comprising:

a first step of forming a solution containing a fluorescent molecule into a fine liquid droplet containing a molecule-group, and causing said fine liquid droplet to adsorbe on a surface of a flat substrate; and

a second step of drying said substrate with said fine liquid droplet stuck to said surface.

8. A method according to claim 7, wherein

said fluorescent molecule in said molecule-group includes a complex of a protein and a predetermined number of substance emitting a fluorescence, or said substance emitting said fluorescence.

9. A method according to claim 8, wherein

said solution containing said fluorescent molecule is prepared by separating a molecule bonded with a predetermined number of said substance emitting said fluorescence from an unbounded molecule by chromatography.

10. A method according to claim 9, wherein

said solution containing said fluorescent molecule is formed in said fine liquid droplet by a ultrasonic wave.

11. A method according to claim 10, wherein

said molecule-group contained in said fine liquid droplet comprises four fluorescent molecules or less on an average.

12. A method according to claim 10,

said first step further comprising a step scattering in an atmosphere said solution containing said fluorescent molecule in said fine liquid droplet, and then causing said fine liquid droplet to adsorb on said surface of said substrate.

13. A method according to claim 7, wherein

said drying in said second step is conducted at a room temperature or said room temperature or less.

14. A specimen prepared by forming a solution containing a fluorescent molecule into a fine liquid droplet containing a molecule-group, sticking said fine liquid droplet to a surface of a substrate, and air-drying said surface.

15. A specimen according to claim 14, wherein

said substrate with said adsorbed molecule-group includes a silicon wafer.

16. A specimen according to claim 15, wherein

said substrate with said adsorbed molecule-group is disk-shaped.

17. A device for determining a location of a molecule-group and the number of a fluorescent molecule in the molecule-group comprising:

a light source for irradiating a excitation light to a local area on a flat substrate with a molecule-group comprising an adsorbed fluorescent molecule; and

fluorescence detecting means for detecting a fluorescence photon generated by irradiating said excitation light to said local area on said substrate, and measuring the number of said fluorescence photon per unit period of time as a quantized fluorescence intensity of said molecule-group.

18. A device according to claim 17, wherein

said fluorescence detecting means is disposed at a position which is out of an optical path of said excitation light irradiated from said light source, and reflected light on a surface of said substrate.

19. A device according to claim 18, wherein

said fluorescence detecting means includes an optical microscope for condensing said fluorescence in said local area on said surface of said substrate; and

a photon counting system for counting the number of a fluorescence photon condensed by said optical microscope, and displaying an appearance frequency distribution of said fluorescence photon on a two-dimensional image corresponding to said local area.

20. A device according to claim 19, wherein

said photon counting system forms an appearance frequency distribution of said fluorescence photon for said unit period of time by plotting a total number of fluorescence photons corresponding to a measuring position of said fluorescent photon on a two-dimensional image corresponding to said local area on said substrate, every time said fluorescence photon is detected by irradiating said excitation light; and

said photon counting system identifies a fluorescent spot where said molecule-group is present, based on said appearance frequency distribution, and determines said molecule number of said fluorescent molecule in said molecule-group, based on the number of said fluorescent photon measured in said fluorescent spot.

21. A device according to claim 19, wherein

said optical microscope can change its magnification.

22. A device according to claim 18, wherein

said excitation light includes a laser beam.

23. A device according to claim 17,

further comprising moving means for relatively displacing said local area on said substrate.

24. A device according to claim 23, wherein

said moving means includes an X-Y stage being movable horizontally to said fluorescence detecting means, and a rotary stage supported by said X-Y stage for fixing said substrate.

25. A method for determining a sequence of bases in nucleic acid comprising:

a first step of irradiating a pulsed excitation light to a local area where a base is present, on a flat substrate with said bases of a nucleic acid in a sequence;

a second step of detecting a fluorescence generated from said base present in said local area on said flat substrate, and identifying a kind of said base, based on a wavelength distribution of said fluorescence, and a lifetime of said fluorescence; and

a third step of moving said local area on said flat substrate along said sequence of said base.

26. A method according to claim 25,

said second step further comprising a step of measuring the number of a fluorescence photon per unit period of time generated by irradiating said excitation light to said local area to identify a location of said base in said local area.

27. A method according to claim 25, wherein

an irradiation angle of said excitation light to said flat substrate is set so as to avoid an angle which a reflected light from said irradiated excitation light effects on the detecting said fluorescence photon in said step.

28. A method according to claim 25, wherein

said substrate includes a silicon wafer.

29. A method according to claim 28, wherein

said substrate is disk-shaped.

30. A method according to claim 29, wherein

said flat substrate with said bases stuck to is prepared by falling a liquid droplet containing said

base on said surface of said substrate which is horizontally moved on rotation, while each of said bases is cutting off sequentialy, toward a center along a periphery of said substrate, and drying said surface of said substrate.

31. A method according to claim 30, wherein
said drying is conducted at a room temperature or said temperature or less.

32. A method according to claim 28, wherein
a monoclonal antibody which selectively bonds with a predetermined base is applied to said surface of said substrate.

33. A method according to claim 26, wherein
said detection of said fluorescence from said base stuck to said surface of said substrate is conducted while cooling said base.

34. A device for determining a sequence of bases in nucleic acid comprising:
a light source for irradiating a pulsed excitation light to a local area where a base is present, on a flat substrate with said bases of a nucleic acid in a sequence;
fluorescence detecting means for detecting a fluorescence generated from said base present in said local area on said flat substrate, and identifying a kind of said base, based on a wavelength distribution of said fluorescence, and a lifetime of said fluorescence; and
moving means for moving said local area on said flat substrate along said sequence of said bases.

35. A device according to claim 34, wherein
said fluorescence detecting means includes an optical microscope for condensing said fluorescence in said local area on said surface of said substrate; and
a photon counting system for counting the number of a fluorescence photon condensed by said optical microscope, and displaying an appearance frequency distribution of said fluorescence photon on a two-dimensional image corresponding to said local area.

36. A device according to claim 34, wherein
said fluorescence detecting means is disposed at a position which is out of an optical path of said excitation light irradiated by said light source, and of a reflected light on said surface of said substrate.

37. A device according to claim 34, wherein
said substrate includes a silicon wafer.

38. A device according to claim 37, wherein
said substrate is disk-shaped.

39. A device according to claim 36, wherein
said light source includes a laser device.

40. A device according to claim 34, wherein
said moving means includes an X-Y stage being movable horizontally to said fluorescence detecting means, and a rotary stage supported by said X-Y stage for fixing said substrate.

41. A device according to claim 34, comprises cooling means for cooling said local area on said surface of the substrate.

42. A method of determining the relative position of one or more fluorescent molecules within a molecule-group, comprising the steps of:
applying excitation radiation to a substrate on which is disposed said molecule-group; and
detecting at a plurality of positions fluorescent radiation from the molecules within said molecule-group and determining the position or positions within the molecule-group from which said fluorescent radiation is emitted, thereby to determine the position of said fluorescent molecule or molecules within the molecule-group.

43. A method as claimed in claim 42, wherein said molecule-group comprises at least two different fluorescent molecules having respective fluorescence characteristics and the positions of said molecules within

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the molecule-group are determined by measuring the characteristics of the detected fluorescent radiation.

44. A method of determining the relative positions of molecules in a group, comprising spatially fixing a sample of the molecule-group and detecting the fluorescence of molecules in the group at different positions.

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Fig. I
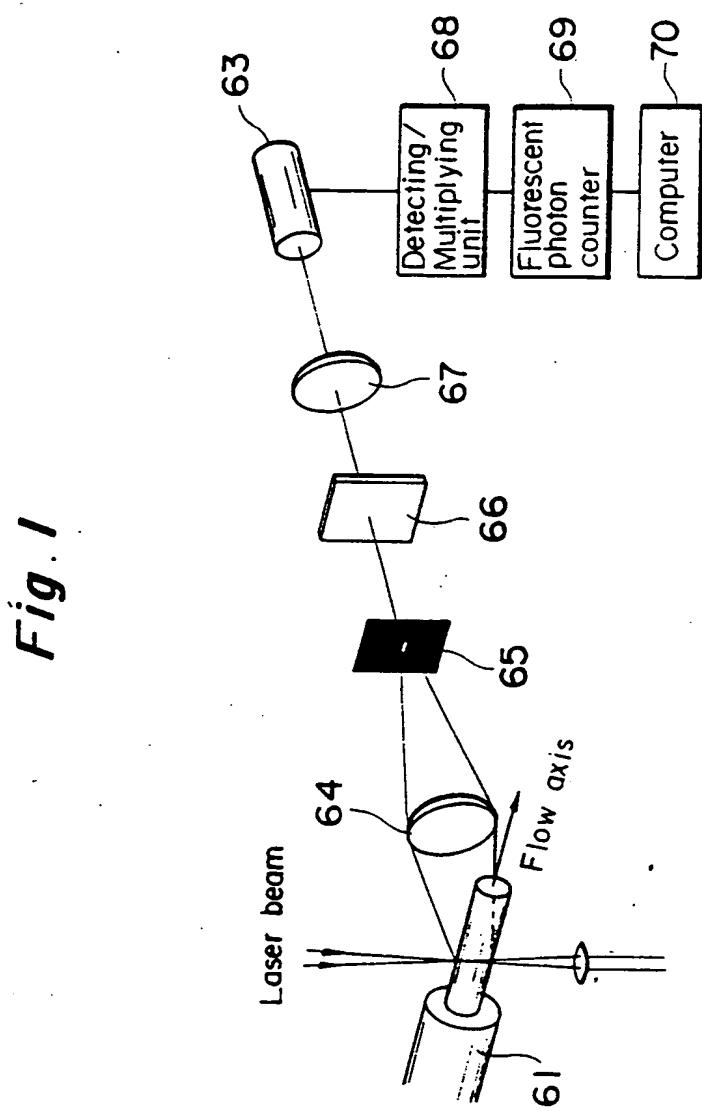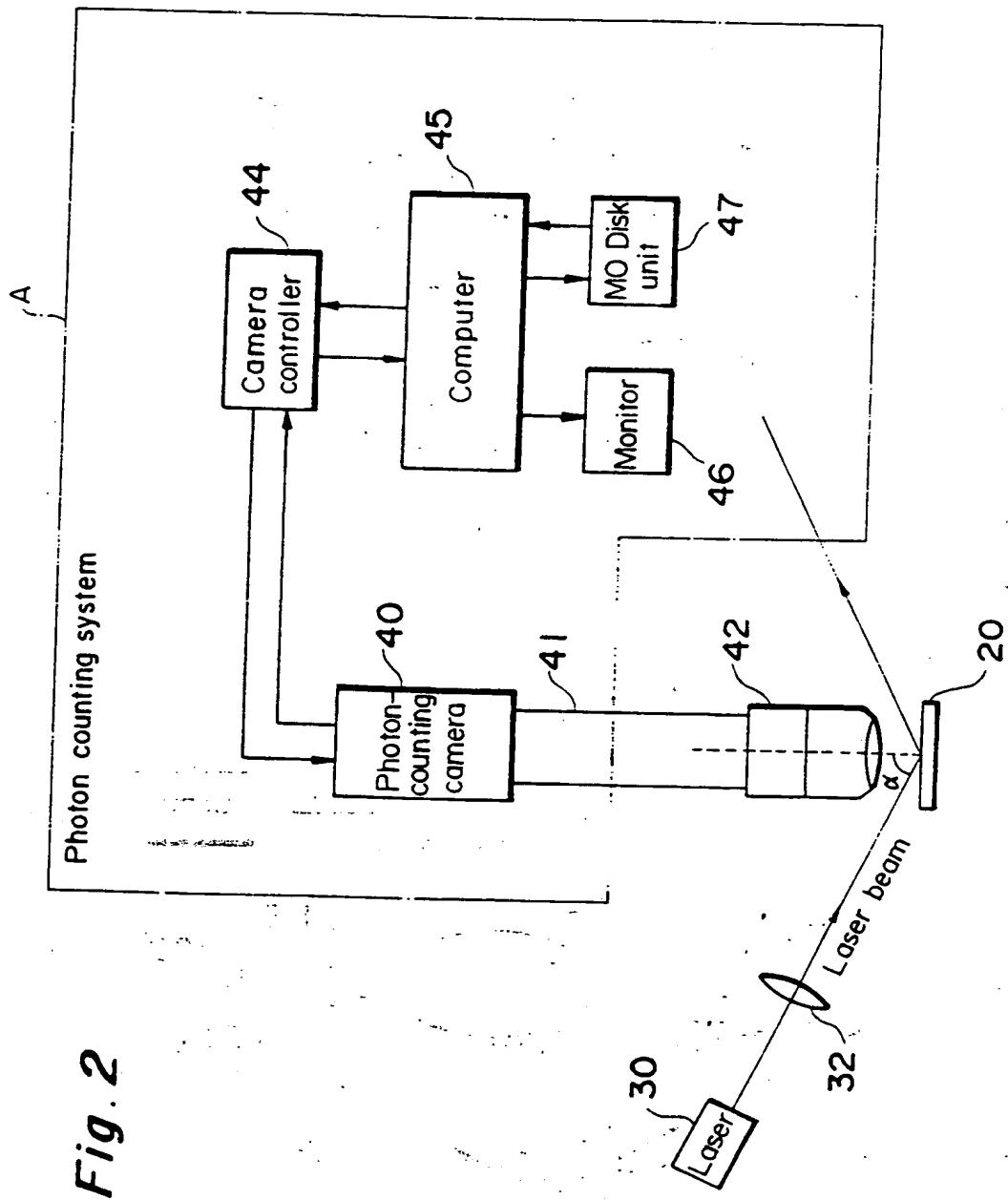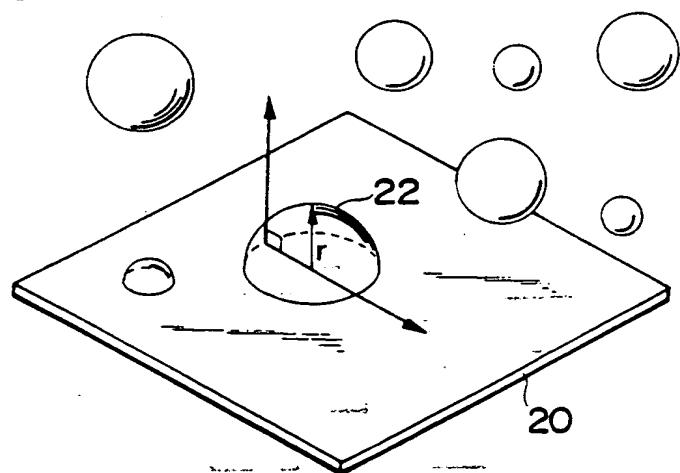
Fig. 2

Fig. 3

Fig. 4
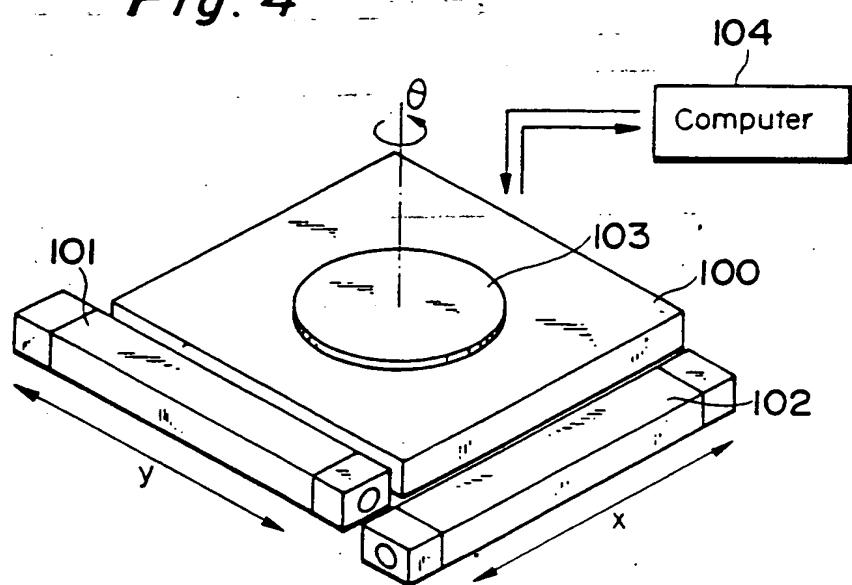
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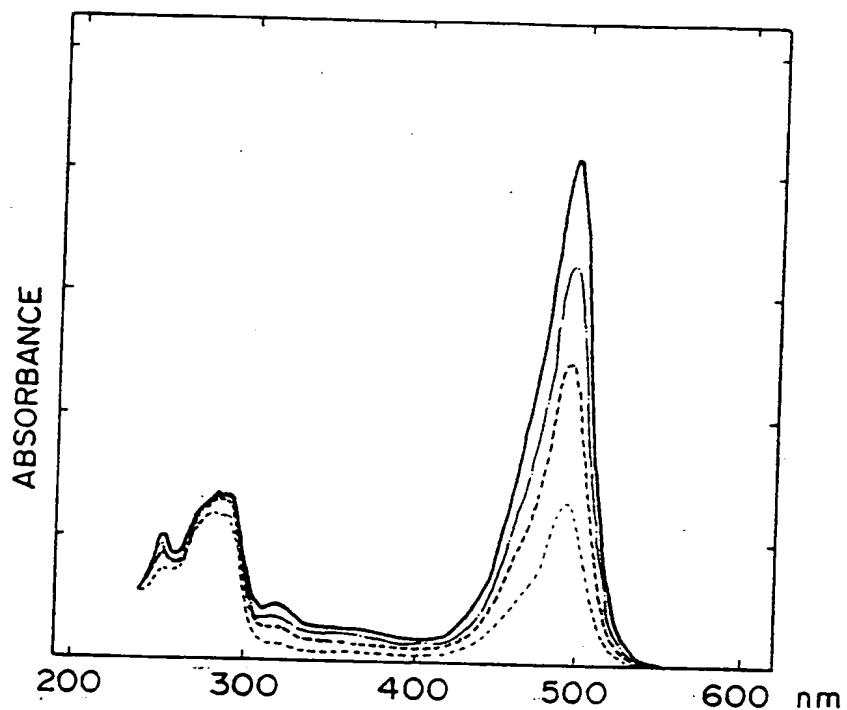
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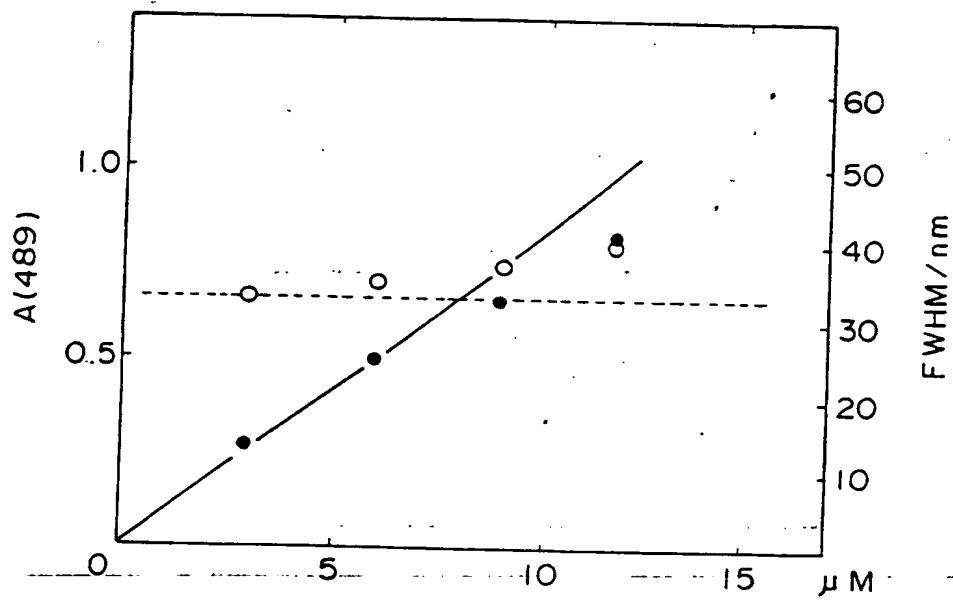
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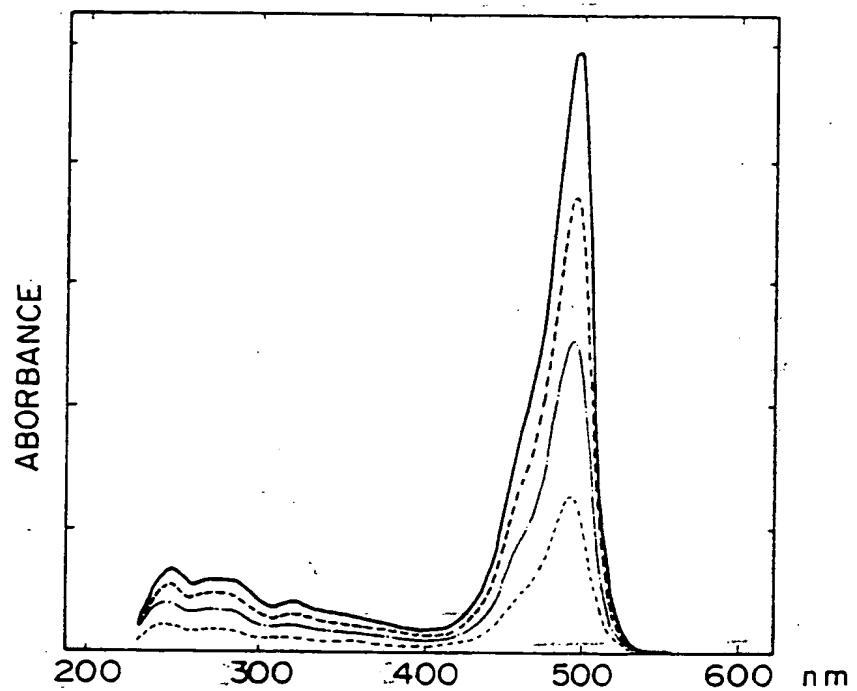
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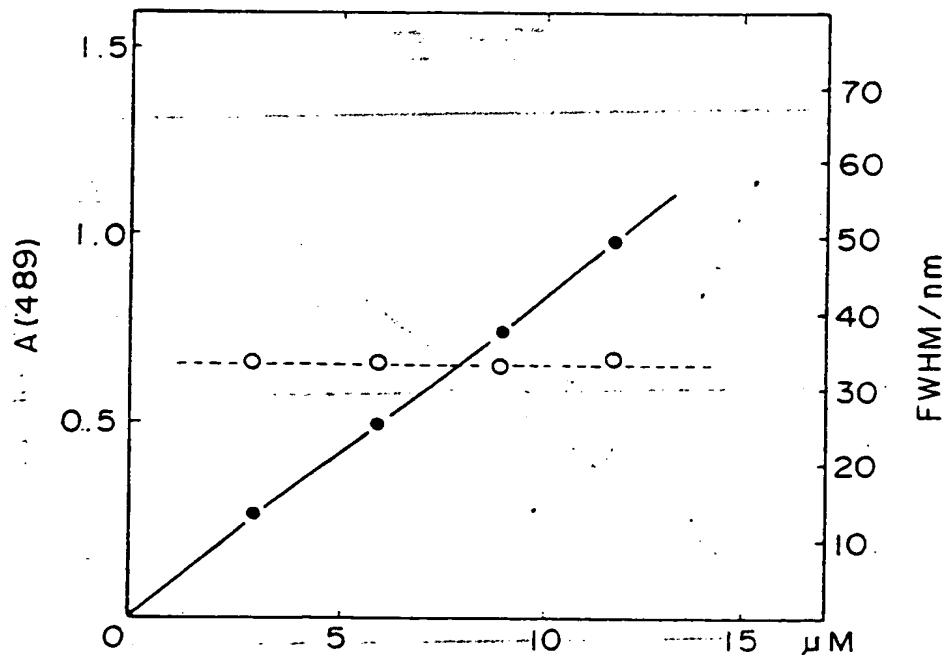
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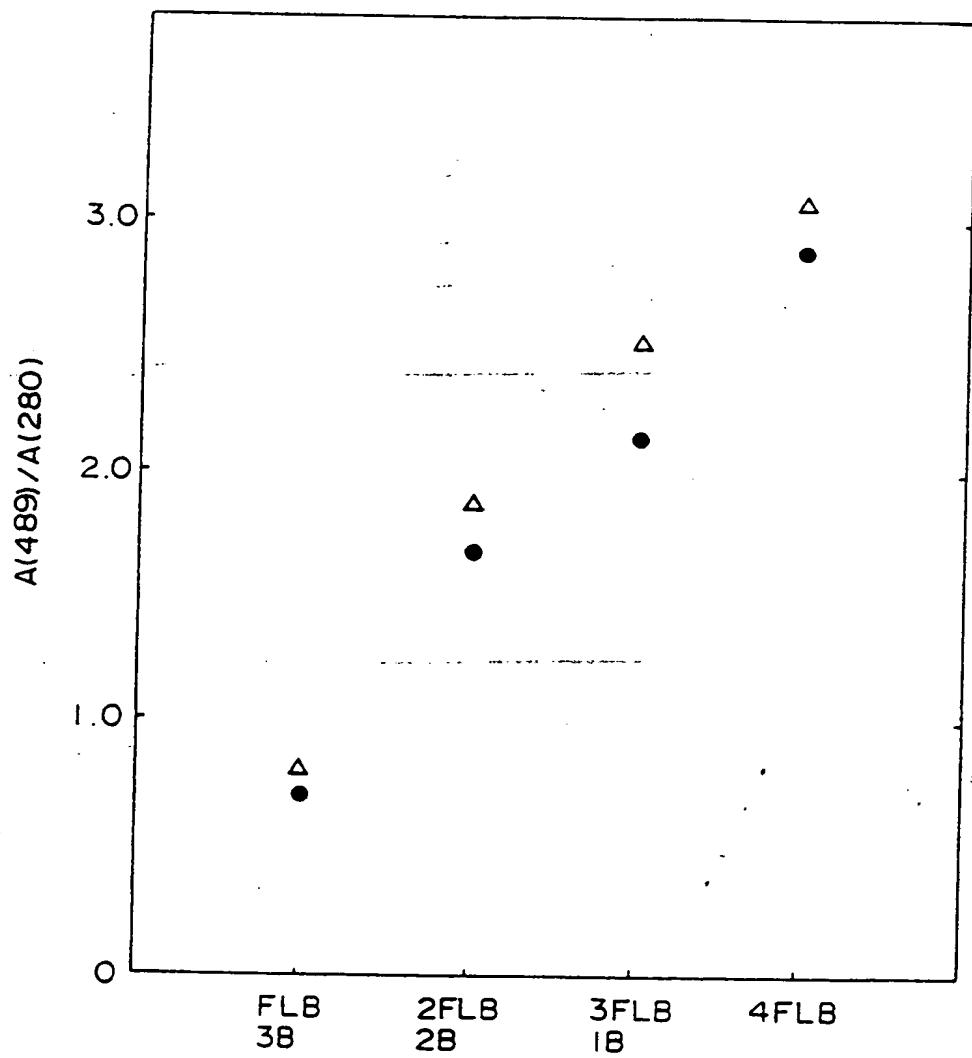
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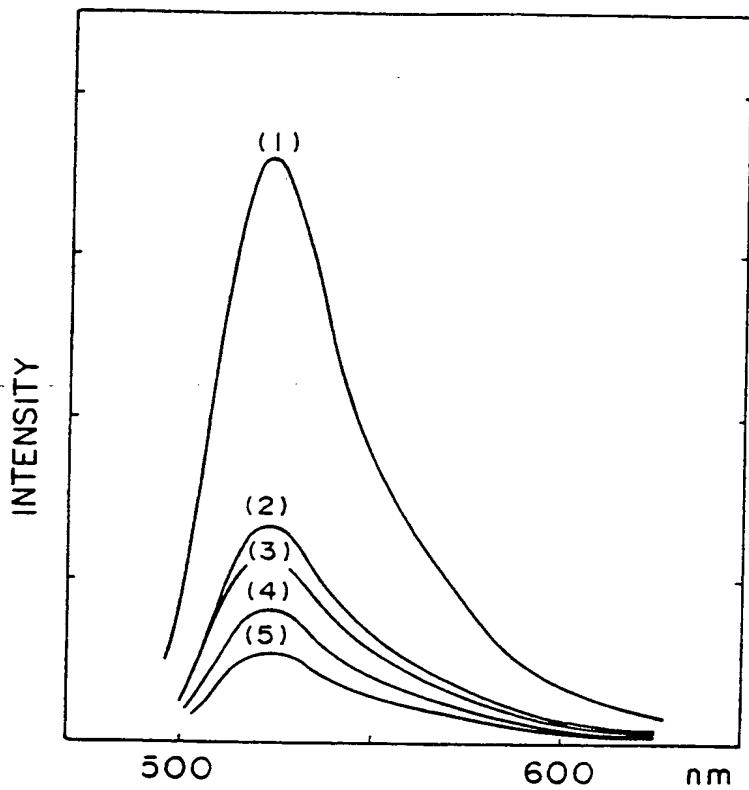
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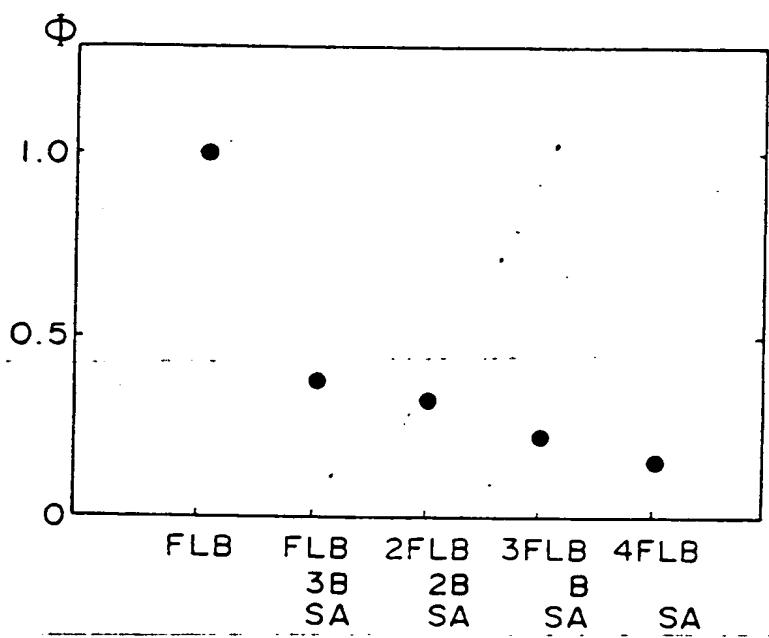
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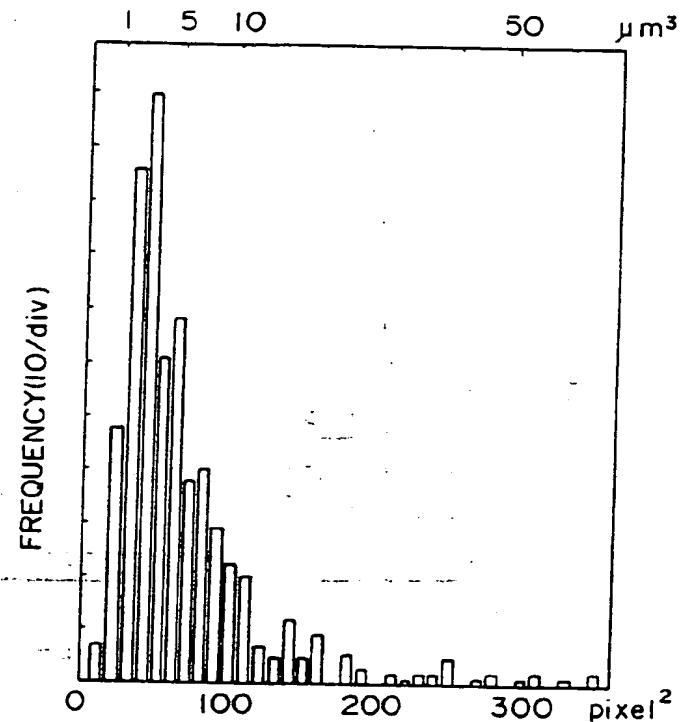
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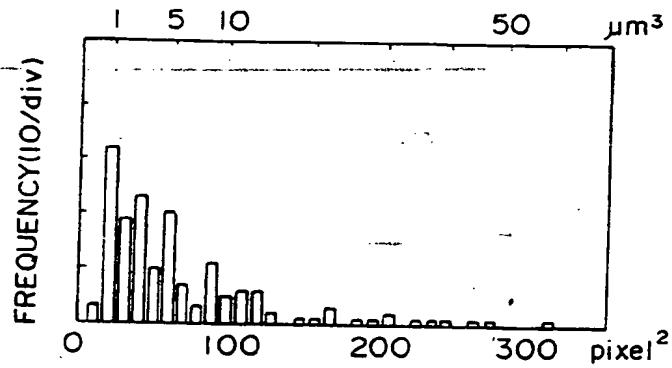
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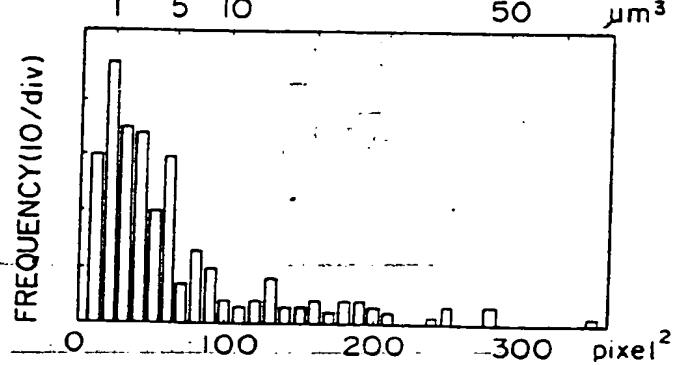
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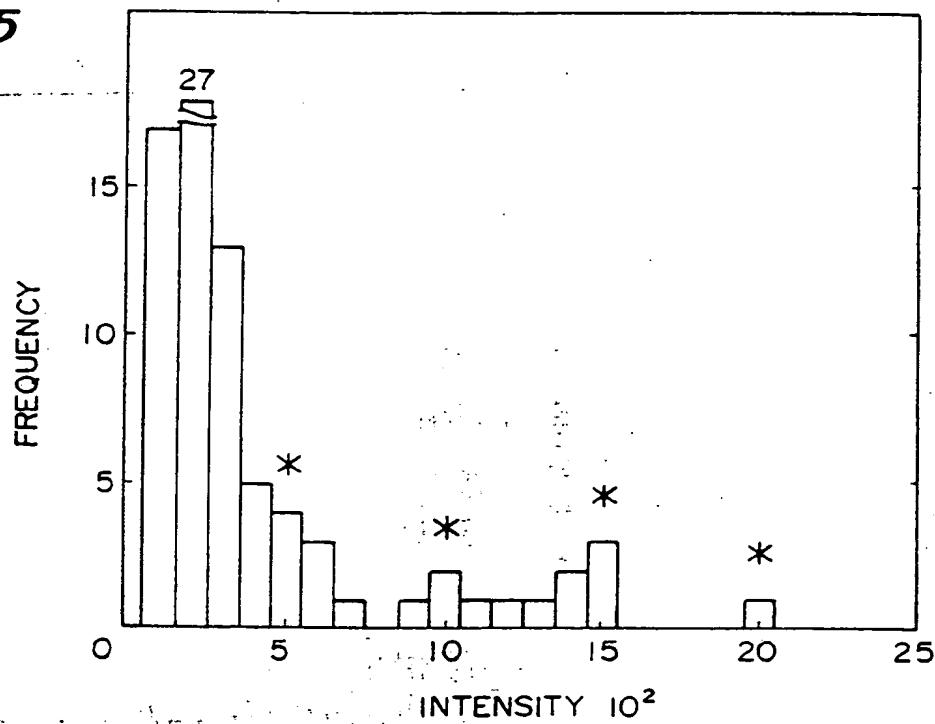
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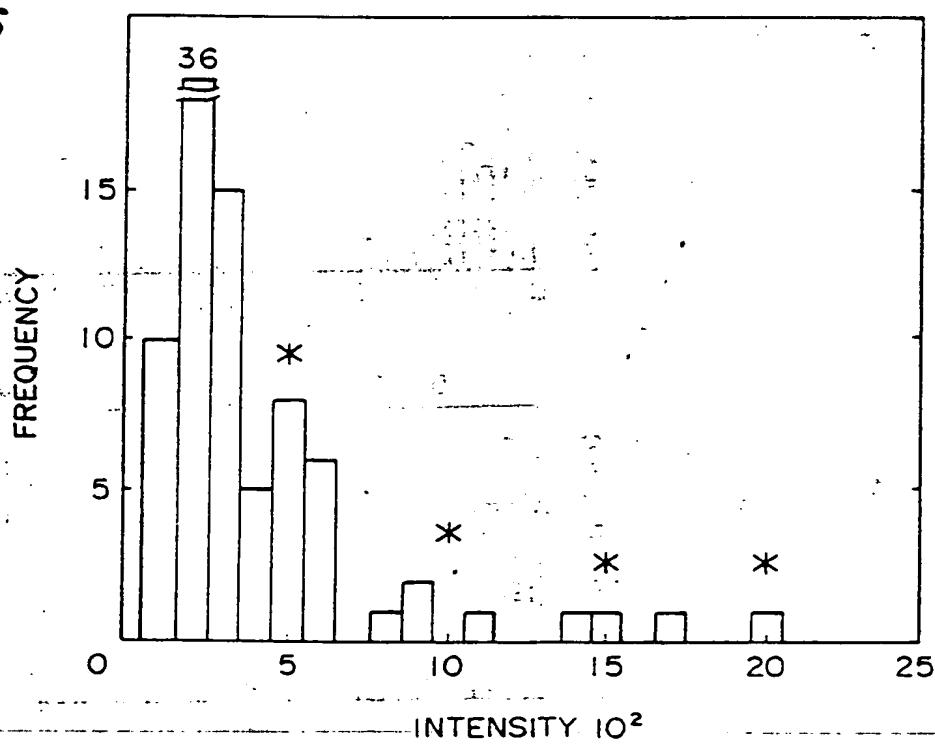
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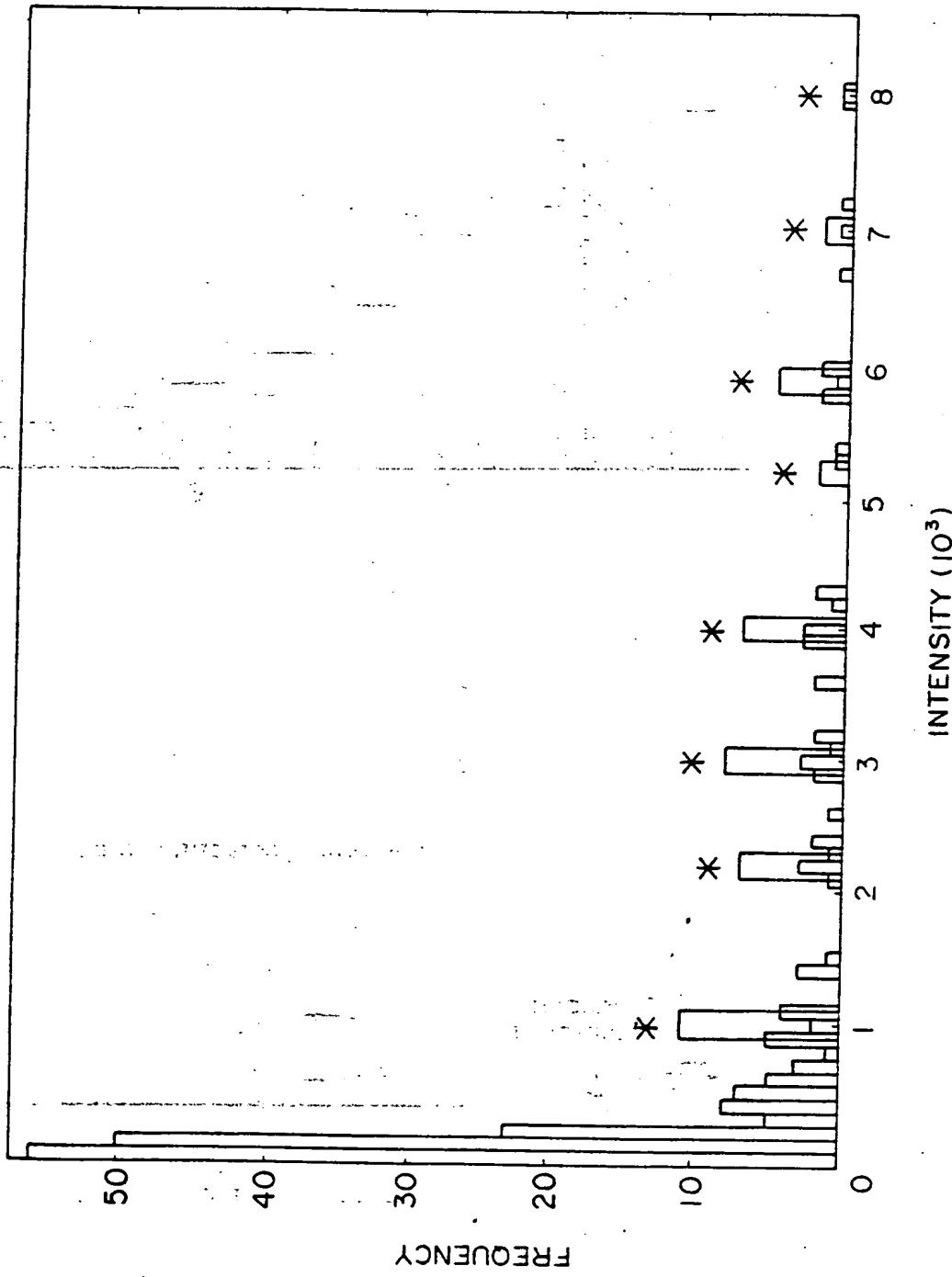
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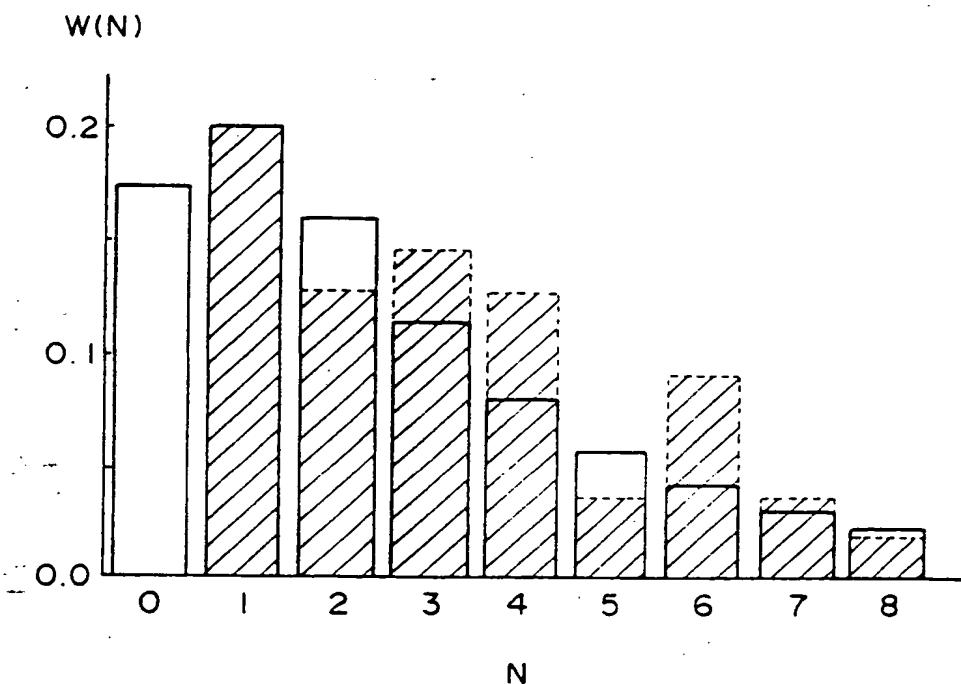
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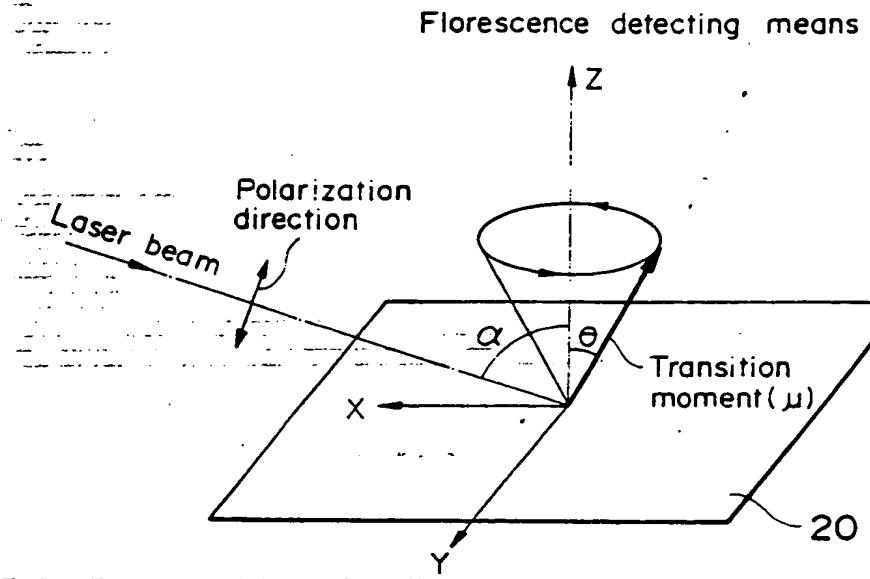
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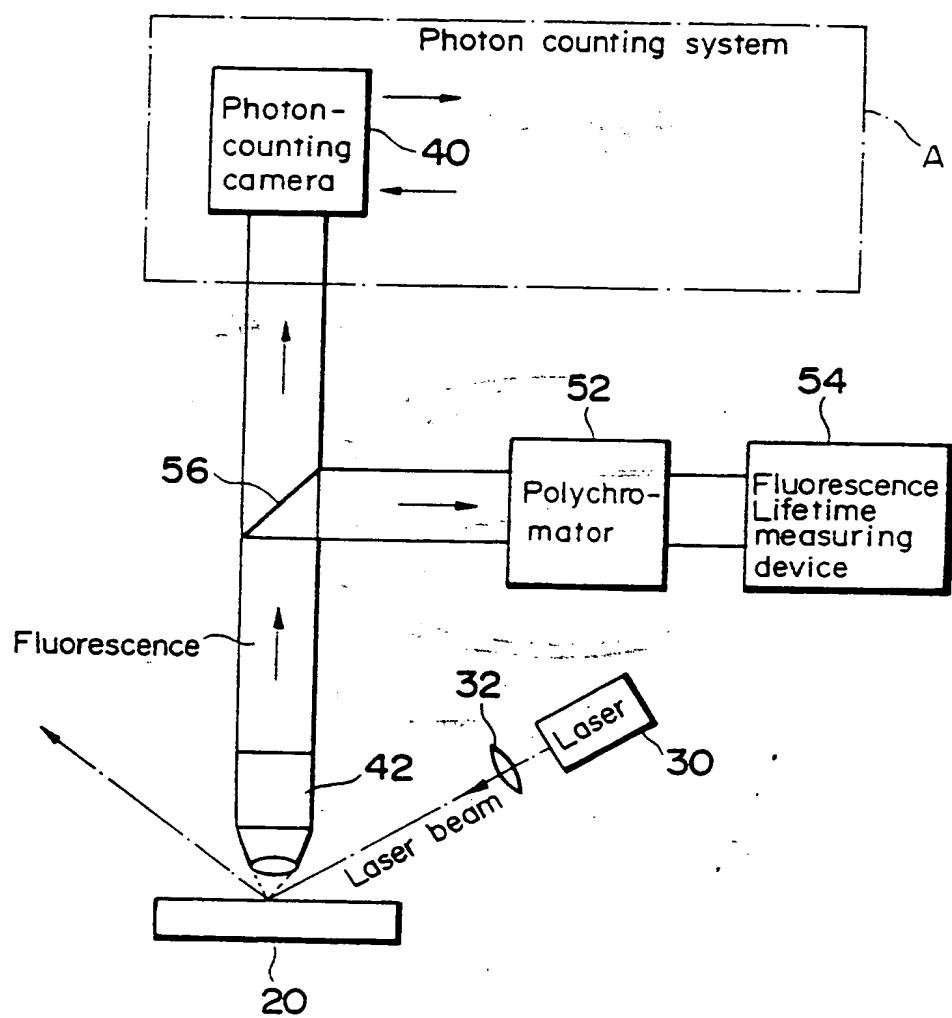
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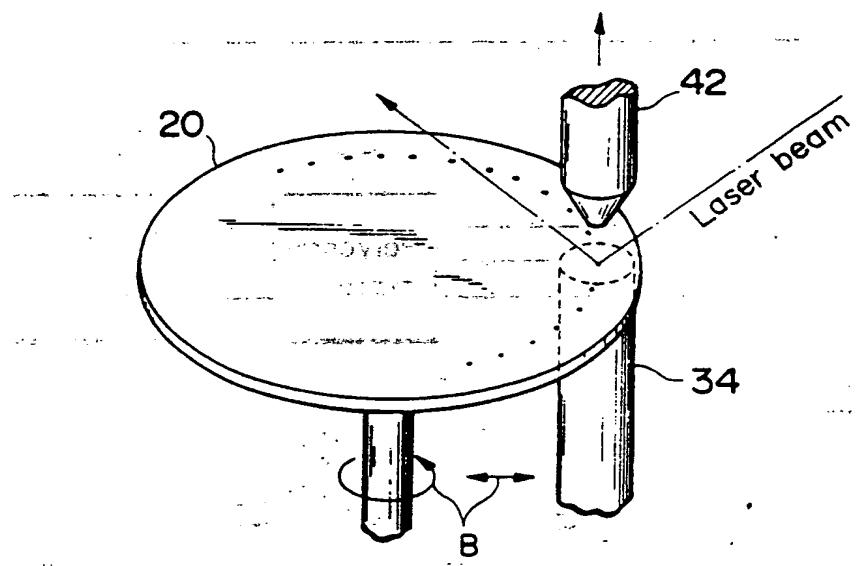
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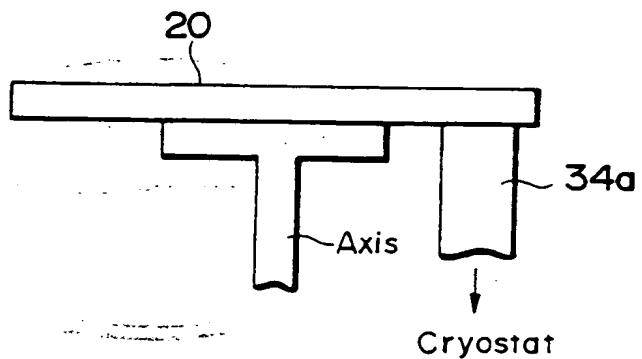
Fig.23

Fig.24

Fig.25

Fig.26

Fig.27

Lequid

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To piezoelectric driver

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Base

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Fig.28

To photon -
counting camera

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Laser beam

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Polychro -
mator 52

Fluorescence
lifetime
measuring 54
device

Fig . 29

Fig . 30

Enzyme

Fig . 31

Fig . 32

Luminescence reagent

Enzyme

Luminescence

Base

20

Luminescence reagent

Enzyme

Luminescence

20

| | European Patent Office | EUROPEAN SEARCH REPORT | Application Number
EP 93 30 8986 |

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
X	WO-A-90 14589 (LELAND STANFORD JUNIOR UNIVERSITY) * abstract *	1-3,17, 23,42,44	G01N21/64 G01N15/14
Y	* page 1, line 18 - line 24 *	4,18,25, 34,43	//C12Q1/68
A	* page 10 - page 12 * * figures 9-11 *	5,6,19, 20, 22-24, 26,35, 39,40	

Y	US-A-5 062 942 (KAMBARA) * column 5, line 10 - line 22 *	4,18,43	
A	* column 8, line 49 - line 66; figure 3A *	27,36	

Y	CHEMICAL PHYSICS LETTERS vol. 173, no. 1 , 28 September 1990 pages 129 - 131 B. BROCKLEHURST 'Luminescence of DNA excited in the vacuum ultraviolet'	25,34	
A	* abstract; figure 1 *	27,36	TECHNICAL FIELDS SEARCHED (Int.Cl.5)
	---		G01N
A	EP-A-0 440 342 (UNIVERSITY OF CALIFORNIA)	1-3, 22-26, 34,39, 40,42	B01L
	* abstract * * column 1, line 13 - line 17 * * column 3, line 43 - line 54 * * column 4, line 11 - line 48 * * column 5, line 15 - line 31 * * column 5, line 44 - line 48 * * column 6, line 10 - line 25 * * column 7, line 13 - line 17 * * column 7, line 22 - line 27 * * claim 7; figures 1-4,6 * ---		
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	22 February 1994	Thomas, R.M.

EUROPEAN SEARCH REPORT

European Patent Office

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
X	WO-A-92 18608 (AMERSHAM INTERNATIONAL) * abstract * * page 1, paragraph 1 * * page 3, line 9 - line 17 * * page 11, line 2 - line 13 * * page 11, line 27 - line 30 * * page 12, line 15 - line 19 * * page 13, line 3 - line 7 * * page 13, line 27 - line 34 * * page 14, line 33 - page 15, line 10 * * page 20, line 20 - line 32 *	7,8,13, 14	
A	* claim 1; figures 1,2 *	10-12, 30-32	
A	EP-A-0 469 444 (BOEHRINGER MANNHEIM) * page 2, paragraph 1 -paragraph 3 * * page 4, line 28 - line 30 * * page 6, line 55 - line 58; figure 1 *	7,14	
D,A	US-A-4 962 037 (JETT) * abstract *	7,14	TECHNICAL FIELDS SEARCHED (Int.Cl.5)
P,X	EP-A-0 541 296 (HAMAMATSU) * column 3, line 46 - line 55 * * column 4, line 40 - line 43 * * column 4, line 51 - line 53 *	1-4,17, 18,23,42	
P,A	* column 5, line 4 - line 7; figure 2 *	5-12,14, 25,27, 34,36	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	22 February 1994	Thomas, R.M.